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(54) Title: METHODS FOR PREPARING AND USING IMMORTALIZED HUMAN NEUROENDOCRINE CELLS

(57) Abstract

Disclosed are methods of generating immortalized human regulated secretory cells and cell lines of neuroendocrine origin, the immortalized human neuroendocrine cells and cell lines thus generated, and methods of using these human cells, for example, in the production of secretory polypeptides and in cell-based therapeutic methods. Exemplary cells are stable human β-cells that produce insulin. The immortalized human neuroendocrine cells and cell lines are generally prepared by methods using promoters, and preferably tissue-specific promoters, to express immortalizing constructs and, in certain embodiments, by using modified promoters, combinations of immortalizing constructs, viral delivery and defined culture media.

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DESCRIPTION

METHODS FOR PREPARING AND USING IMMORTAIZED HUMAN NEUROENDOCRINE CELLS

BACKGROUND OF THE INVENTION

The present application claims the priority date of co-pending provisional application Serial No. 60/091,105 (Attorney Docket No. UTSD:538PZ2 (BTGN:020PZ2)), filed June 29, 1998, and provisional application Serial No. 60/071,209 (Attorney Docket No. UTSD:538PZ1 (BTGN:020PZ1)), filed January 12, 1998, the entire disclosures of which are incorporated herein by reference without disclaimer. The U.S. Government owns rights in the present invention pursuant to grant number DK50610 from the National Institutes of Health.

1. Field of the Invention

The present invention relates generally to the field of human cell biology. More particularly, it provides methods for preparing immortalized human cells and cell lines of neuroendocrine origin, such as human β-cells, and the cells and cell lines thus produced. The methods for preparing the cells are generally based on the expression of an immortalizing construct and, in certain embodiments, include the use of cell-specific expression, multiple constructs, modified promoters, viral delivery and defined culture media. Methods of using the immortalized human cells, for example, in the production of secretory polypeptides, drug discovery, diagnostic applications such as continuous, non-invasive glucose monitoring and in cell-based delivery methods are also provided.

2. Description of Related Art

Cells of neuroendocrine origin generally have the capacity to synthesize and secrete one or more polypeptide products in a regulated manner. For example, cells of the anterior or intermediate lobes of the pituitary produce growth hormone or proopiomelanocortin (POMC)-derived peptides, such as ACTH and MSH; thyroid C cells secrete calcitonin; and distinct types of pancreatic cells produce and secrete hormones such as glucagon and insulin.

Neuroendocrine cells by definition have sorting mechanisms, whereby a given polypeptide or protein, destined for secretion, is targeted to the regulated secretory pathway or the default constitutive secretory pathway. These cells also have processes for achieving secretory protein maturation, which generally involves protein folding, disulfide bond formation, glycosylation, endoproteolytic processing as well as other types of post-translational modifications. Neuroendocrine cells also exhibit controlled release of the secretory protein or polypeptide, most often in response to one or more external signaling molecules, or "secretagogues", and thus have regulatory pathways allowing the cells to secrete a desired product from the secretory storage granules in response to physiological or pharmacological stimuli.

One of the more well known examples of neuroendocrine cells are the β-cells of the islets of Langerhans in the pancreas, which cells secrete insulin in response to secretagogues such as amino acids, glyceraldehyde, free fatty acids, and, most prominently, glucose. The capacity of normal islet β-cells to sense a rise in blood glucose concentration and to respond to elevated levels of glucose by secreting insulin is critical to the control of blood glucose levels. Increased insulin secretion in response to a glucose load prevents chronic hyperglycemia in normal individuals by stimulating glucose uptake into peripheral tissues, particularly muscle and adipose tissue.

Individuals in whom islet β-cell function is impaired suffer from diabetes. Insulin-dependent diabetes mellitus, IDDM (also known as Juvenile-onset or Type I diabetes) represents approximately 10% of all human diabetes. IDDM is distinct from non-insulin dependent diabetes (NIDDM) in that only IDDM involves specific destruction of the insulin producing β-cells of the islets of Langerhans. The destruction of β-cells in IDDM appears to be a result of specific autoimmune attack, in which the patient's own immune system recognizes and destroys the β-cells, but not the surrounding α-cells (glucagon producing) or δ-cells (somatostatin producing) that comprise the islet.

Treatment for IDDM is still centered around self-injection of insulin once or twice daily, and the development of new therapeutic strategies is highly desirable. The possibility of islet or pancreas fragment transplantation has been investigated as a means for permanent insulin replacement (Lacy, 1995). However, this approach has been severely hampered by the difficulties associated with obtaining tissue, as well as the finding that transplanted islets are recognized and destroyed by the same autoimmune mechanism responsible for destruction of the patients' original islet β-cells.

Loss or impaired function of other cells of neuroendocrine origin is associated with other human diseases and disorders. For example, the failure of substantia nigra cells to properly produce dopamine results in Parkinson's Disease, the failure of thyroid cells to properly produce thyroid hormones results in athyrotic cretinism, and the loss of adrenal gland cells, with the consequent failure to produce adrenal hormones, results in Adison's Disease. Although diseases such as short stature, Paget's Disease, infertility and endometriosis are generally treated by recombinant growth hormone, calcitonin, gonadotropins, and gonadotropin-releasing hormone, respectively, this again is far removed from an adequate treatment strategy as it relies upon injections and suffers from the drawbacks of proper dosing, side effects, and patient compliance.

U.S. Patent 5,427,940 provided, for the first time, recombinant cells that secrete insulin in response to glucose. The generation of such artificial β-cells is achieved through the introduction of one or more genes selected from the insulin gene, the glucokinase gene and the GLUT-2 glucose transporter gene, so as to provide an engineered cell having all three of these genes in a biologically functional and glucose-responsive configuration.

The availability of the engineered cells of U.S. Patent 5,427,940 makes cell-based insulin replacement therapy for IDDM a realistic goal. However, while evidently of significant use, this engineering technology has, to date, been most successful in connection with cells of non-human origin. Furthermore, the provision of the glucose-sensing, insulin secreting gene combination, whilst pioneering in the field of diabetes, does not adequately address losses or deficiencies in other neuroendocrine cell systems.

It can thus be seen that the art has needed immortalized human neuroendocrine cells and cell lines for some time. Indeed, a number of attempts to generate human β-cells have been reported, but all to date have at best produced "immortal-like" cells that, while able to remain in culture for multiple generations, tend to lose certain necessary functional properties over time, particularly the regulated secretion of the desired polypeptide. For example, different culture conditions (Beattie *et al.*, 1991; Hayek *et al.*, 1995; PCT application, WO 95/29989), viral transformation (Neisor *et al.*, 1979; Levine *et al.*, 1995; U.S. Patent 4,332,893), cell fusion (U.S. Patent 4,195,125; PCT application WO 87/05929) and expression of transfected oncogenes (Soldevila *et al.*, 1991; WO 91/09939) have failed to produce immortalized insulin-secreting human cells that retain regulated insulin-secreting ability over time.

The ultimate development of methods for creating immortalized human neuroendocrine cells and cell lines that retain the desired neuroendocrine properties, such as regulated secretion of a desired polypeptide, and the ability to engineer desired functions or properties into these immortalized human neuroendocrine cells, given the long-standing need and the many reported failures, would thus represent a significant advance in the art.

SUMMARY OF THE INVENTION

The present invention overcomes these and other drawbacks inherent in the prior art by providing methods for the production of immortalized human cells and cell lines of neuroendocrine origin. The cells of the invention may be used, for example, in the *in vitro* production of secreted polypeptides, in drug screening and discovery applications, in diagnostic applications such as continuous, non-invasive glucose monitoring and in the development of *in vivo* cell-based delivery of proteins and polypeptides. The immortalized cells of the present invention are functionally defined as having maintained a regulated secretory pathway, as being stable to *in vitro* culture and, in certain embodiments, as being amenable to further engineering.

Accordingly, the present invention provides methods for preparing immortal human neuroendocrine cells. The immortal human neuroendocrine cells of the invention will generally be characterized as comprising at least a first immortalizing genetic construct under the control of at least a first promoter specific for the neuroendocrine cell, wherein the cell secretes at least a first polypeptide. Thus, in a most general sense, the method comprises providing to a non-immortal human neuroendocrine cell that secretes at least a first polypeptide an effective amount of at least a first immortalizing product.

While in certain aspects of the invention a purified or isolated human neuroendocrine cell will be utilized as the starting non-immortalized cell, in many aspects of the present invention, the non-immortal human neuroendocrine cells will be present in a heterogeneous, relatively heterogeneous, partially purified, or pre-selected cell population that comprises the non-immortal human neuroendocrine cells. Thus, in a further aspect of the present invention, the method comprises providing to a cell population comprising non-immortal human neuroendocrine cells an effective amount of at least a first immortalizing genetic construct comprising at least a first immortalizing expression unit under the transcriptional control of a promoter specific for the neuroendocrine cells, the at least a first immortalizing genetic construct specifically expressing at least a first immortalizing product in the non-immortalized human neuroendocrine cells within the cell population.

The present invention also provides methods for preparing immortal human neuroendocrine cells, generally characterized as comprising providing to a non-immortal human neuroendocrine cell an effective amount at least a first immortalizing genetic construct comprising at least a first telomerase catalytic component under the control of at least a first promoter that expresses the at least a first telomerase catalytic component in the cell, wherein the cell secretes at least a first polypeptide. In certain preferred embodiments, the promoter is specific for the neuroendocrine cell.

In certain embodiments, after immortalization the immortalizing genetic construct will be excised from the genome of the cell, and thus separated from the cell, by use of a molecular biological excision system, for example, such as the Cre/Lox site-specific recombination system or by the use of the recombination activating genes, RAG 1 and RAG 2, in conjunction with the use of specific recombination signal sequences (RSSs).

It is generally required that the promoter or promoters for use in driving expression of the immortalizing genetic constructs will cause expression of the constructs to a level effective to immortalize the host human neuroendocrine cell, and in certain aspects the promoter will be specific for the host human neuroendocrine cell. Therefore, the cells of the invention may be characterized as stable human neuroendocrine cells which comprise at least a first immortalizing genetic construct under the control of a promoter, in certain embodiments a promoter that is specific for the neuroendocrine cell, wherein the promoter expresses the at least a first immortalizing genetic construct to a degree sufficient to effect immortalization of the cell, and wherein the cell secretes at least a first polypeptide.

In certain embodiments, it is not required that the promoter be "specific" in the context of being naturally expressed only in the one neuroendocrine cell that is to be immortalized by the methods of the invention. In these embodiments all that is required is that the promoter be "context specific", in that the promoter will be present only in the cell types to be immortalized and not within other cell types in a population of starting cells. For example, in a population of starting cells for the intended generation of an immortal, stable human β-cell, the use of one or more promoters that are expressed in tissues other than β-cells is contemplated so long as those promoters chosen are not capable of directing expression in other cell types within the population of starting cells, particularly those cells such as fibroblasts, ductal cells, and the like.

In certain embodiments the immortal human neuroendocrine cells of the invention will be stable cells that have maintained a functional, regulated secretory pathway, wherein the cells secrete one or more polypeptides *via* the intact secretory pathway. In certain aspects of the present invention, the immortal human neuroendocrine cells will preferably secrete at least a first endogenous secretory polypeptide or peptide. In other aspects the immortal human neuroendocrine cells will preferably secrete at least a first exogenous secretory polypeptide or peptide, or at least a first endogenous or exogenous polypeptide or peptide, preferably a bioactive polypeptide or peptide, that is not normally secreted from the human neuroendocrine cell. In embodiments involving secretion of at least a first exogenous polypeptide or peptide, the cell may be provided with at least a first exogenous gene that encodes at least a first exogenous polypeptide or peptide, leading to secretion of at least a first exogenous polypeptide or peptide by the cell. In particular aspects involving secretion of one or more exogenous polypeptide(s), the expression of a gene encoding at least a first endogenous polypeptide is inhibited in conjunction with providing the cell at least a first gene encoding at least a first exogenous polypeptide.

In certain embodiments, the immortal human neuroendocrine cells of the invention may be characterized as human cells that have a functional, regulated secretory pathway, wherein the cells comprise at least a first immortalizing genetic construct, such as a telomerase catalytic subunit, under the control of a promoter, or a promoter that is specific for the cell, wherein secretion of at least a first polypeptide from the cells is regulated or modulated in response to at least a first selected modulator. In a most general sense, the term "secretagogue" will be used for modulators of secretion that lead to increased secretion of a polypeptide from a cell, while the term "inhibitor" will be used for modulators of secretion that lead to decreased secretion of a polypeptide from a cell. However, as discussed herein in detail, it is not a requirement that the immortalized cells maintain their physiological secretory responses to one or more modulator, as such responses may be re-engineered into the resultant immortal cells. In preferred aspects of the present invention, secretion of at least a first polypeptide from the immortal human neuroendocrine cells is regulated or modulated in response to glucose.

Preferred characteristics of the cells of the invention will be that they are culturable and will be capable of growing *in vitro*, which cells may thus be described as "immortal cells". It will also be generally required that the cells of the invention maintain at least a minimum differentiated phenotype, and as such the human neuroendocrine cells of the invention may be characterized as differentiated human cells having a regulated secretory pathway and comprising at least a first immortalizing genetic construct under the control of one or more promoter, or preferably a promoter specific for the secretory cell, wherein the cells maintain a differentiated phenotype after immortalization and secrete at least a first polypeptide.

The sources of starting materials to provide the population of starting cells for use in the present invention include, for example, fetal cells, primary cells obtained from human tissues, cells obtained from neuroendocrine cell lines and cells obtained from human neuroendocrine tumors, such as may be obtained from a resected tumor, for example an insulinoma. In certain embodiments, the starting, non-immortalized human neuroendocrine cell is passaged *in vivo*.

Certain preferred cell types and sources of tumors for such cell types are disclosed herein in Table 1. In preferred embodiments, the starting cells will be a population of cells that predominantly include pancreatic β-cells. In other preferred embodiments, the population of starting cells will be enriched for pituitary cells.

In certain aspects of the present invention, the immortal human neuroendocrine cells of the invention will preferably secrete one or more of the secretory polypeptides listed herein in Table 1, Table 2, Table 3, Table 4 or Table 5. Secretory polypeptides that are glycosylated or amidated are preferred in certain aspects of the invention. In other embodiments, a secreted enzyme such as adenosine deaminase, galactosidase, glucosidase, lecithin:cholesterol acyltransferase (LCAT), factor IX, sphingolipase, lysosomal acid lipase, lipoprotein lipase, hepatic lipase, pancreatic lipase related protein, pancreatic lipase or uronidase are preferred. In particular embodiments, immortal human neuroendocrine cells that secrete leptin, LCAT, or a hormone, such as somatostatin, are preferred.

Immortal human β-cells that secrete insulin will be preferred in certain aspects of the invention, with cells that secrete biologically active, correctly processed human insulin being more preferred. The human insulin may be expressed from an endogenous human insulin gene, or in certain aspects from an exogenous human insulin gene. The immortal human β-cells of the invention may also be advantageously used to secrete endogenous human amylin. Another preferred cell type of the invention, pituitary cells, may be advantageously used to secrete endogenous human growth hormone, ACTH, leuteinizing hormone, follicle stimulating hormone or MSH.

In addition to pancreatic β-cells and pituitary cells, further cells within Table 1 that are more preferred for use in the present invention include thyroid C cells, which secrete endogenous human calcitonin; intestinal endocrine cells, which secrete endogenous human GLP-1 and GIP; and pancreatic α-cells, which secrete endogenous human glucagon.

The immortalizing genetic constructs for use in generating the immortal human neuroendocrine cells of the invention may comprise one or more genes, cDNAs or other coding region or transcriptional units that encode any protein, polypeptide or peptide that effects immortalization of a cell when expressed or overexpressed in the cell. Exemplary immortalizing constructs include those that express growth factors, such as insulin, hepatocyte growth factor (HGF), platelet derived growth factor (PDGF), nerve growth factor (NGF), growth hormone or epidermal growth factor (EGF), and growth factor receptors, such as an insulin, insulin-like growth factor 1 (IGF-1), HGF, PDGF, growth factor or EGF receptor.

Additional exemplary immortalizing genes, certain of which are herein termed "proto-oncogenes", "oncogenes" or "mutant tumor suppressors", are disclosed herein in Table 8. Table 8 generally categorizes the various oncogenes and mutant tumor suppressors, hereafter termed "oncogenes" for simplicity, that may be used in the invention according to functional criteria defining their effects on a cell.

In certain preferred embodiments, the immortal human neuroendocrine cells of the invention will comprise, or will have comprised during their generation, at least a first immortalizing genetic construct that comprises two operative immortalizing units or oncogenes, or three immortalizing operative units or oncogenes, or in certain embodiments, may even comprise four or five separate immortalizing oncogenes or more.

In embodiments where more than one immortalizing oncogene is used, it may be preferable to include distinct oncogenes that fall into different functional categories. For example, a combination of one oncogene that expresses a protein that perturbs signal transduction may be used in conjunction with an oncogene that expresses a protein that affects or alters the cell cycle control mechanisms. Similarly, any two or more oncogenes selected from the diverse groups of oncogenes that perturb signal transduction, affect cell cycle, alter nuclear transcription, inhibit apoptosis, or that fail to promote apoptosis may be used; as may components of telomerase, such as the catalytic subunit (TERT), and one or more other oncogenes that express immortalizing proteins with pleiotropic activities.

Where an immortalizing oncogene is used to express a protein that perturbs signal transduction, the use of a gene that expresses a $G_{s\alpha}$ protein with an activating point mutation or a mutant form of ras will be preferred. In certain embodiments, preferred oncogenes that alter cell cycle control include those that express a cyclin D1 protein. Preferred oncogenes that alter nuclear transcription include E2F and the *myc* family oncogenes that express Myc proteins; apoptosis inhibiting oncogenes preferably include members of the *bcl* gene family, such as *bcl-2*; and mutant tumor suppressor oncogenes preferably include mutant p53, p21, menin and mutant Retinoblastoma (Rb) genes.

In certain embodiments, the expression of an oncogene that encodes an immortalizing viral protein exemplified by, but not limited to, the SV40 or polyoma large T antigens is particularly contemplated. In such embodiments, it will generally be preferred that the immortalizing genetic construct comprises a temperature sensitive gene that expresses a temperature sensitive large T antigen, allowing for the expression of the construct to be regulated in a temperature-dependent manner.

In other preferred embodiments of the present invention the immortal human neuroendocrine cells will comprise, or will have been generated *via* the provision of, at least a first genetic construct that comprises at least a first growth factor or growth factor receptor gene. In certain embodiments, the growth factor or growth factor receptor genes may themselves act as the immortalizing genetic construct. In this context, although growth factors and growth factor receptors *per se* are not oncogenes, when used in the manner disclosed herein, these genes will be used to effect immortalization of the target cell.

In certain embodiments, the expression, or preferably the overexpression, of at least a first growth factor gene will result in the synthesis and secretion of at least a first growth factor into the extracellular environment. The growth factor will then act in a paracrine manner, in order to stimulate the target cell by binding to an expressed cell surface receptor.

In other embodiments, the expression or overexpression of at least a first growth factor receptor gene may be used in a similar manner to increase the numbers of growth factor receptors expressed by the cell, such that the provision of growth factors in the media will promote immortalization, or at least will provide an added stimulus towards immortalization. In certain embodiments, it is contemplated that both at least a first growth factor gene and at least a first growth factor receptor gene may be provided to the same target cell in order to effect immortalization or to generally stimulate the immortalization process. In the embodiments described above, the provision of at least a first growth factor receptor gene is generally preferred.

In certain aspects of the invention, the at least a first growth factor receptor gene may be provided to the cell *via* infection with one type of recombinant virus expressing the growth factor receptor, allowing for further manipulation of the cell by infection with other recombinant viruses expressing one or more genes encoding immortalizing oncogenic products or even further growth factors.

The cell-specific expression of at least a first growth factor receptor will provide the means for effecting specific immortalization of the target human neuroendocrine cells by providing to the extracellular media one or more growth factors that specifically bind to and activate the provided at least a first growth factor receptor. In preferred embodiments, at least a first particular growth factor not normally expressed by the target cell will be provided, allowing for the specific stimulation of only the target cells upon the provision of at least a first exogenous growth factor to the media.

In addition to the foregoing embodiments in which the growth factor, or preferably the growth factor receptor, itself acts as an immortalizing genetic construct, the present invention further contemplates the use of at least a first growth factor or growth factor receptor gene in combination with at least a first immortalizing genetic construct that comprises one or more oncogenes, such as those oncogenes listed in Table 8. In this manner, the growth factor or growth factor receptor gene will provide an added stimulus that supplements the already immortalizing activities of the at least a first oncogenic element expressed specifically in the target cell.

In any of the growth factor or growth factor receptor embodiments, the currently preferred biological components for use in this invention are hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), growth hormone, or even epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF), with the corresponding receptors being the HGF, IGF-1, PDGF, growth hormone, EGF or bFGF receptor genes. In other related aspects of the invention, the preferred components are the chicken growth hormone and the corresponding chicken growth hormone receptor.

In certain aspects of the present invention, the human neuroendocrine cell is grown in defined media, in media comprising at least a first growth factor specific for the neuroendocrine cell, or in defined media further supplemented with at least a first growth factor specific for the neuroendocrine cell. In preferred embodiments, the human neuroendocrine cell is a human pancreatic β-cell and the growth factor is HGF, IGF-1, PDGF, NGF or growth hormone. In additional preferred aspects of the invention, the human neuroendocrine cell is grown in the defined media shown in Table 9.

In other preferred aspects, the human neuroendocrine cell is proliferating prior to provision of at least a first immortalizing genetic construct. Exemplary methods by which the human neuroendocrine cell may be induced to proliferate include growth on a stimulatory cell matrix, growth in contact with a stimulatory growth factor or initial transfection with DNA comprising at least a first gene encoding at least a first stimulatory growth factor. In other aspects, the human neuroendocrine cell is induced to proliferate by initial infection with a virus, such as adenovirus or adeno-associated virus (AAV). In preferred embodiments, the human neuroendocrine cell is induced to proliferate by initial infection with a virus that comprises at least a first gene that induces cellular proliferation, the gene under the control of a promoter specific for the neuroendocrine cell.

In certain aspects of the present invention, the promoter used to drive the immortalizing oncogene expression is a promoter that is specific for the target human neuroendocrine cell, or that provides specific expression in the context of the population of starting cells for use in the invention. In certain aspects of the present invention, the promoter is a human glucagon-like peptide-1 (GLP-1) or alpha-glycoprotein promoter (GenBank Accession Number L05632). Other exemplary promoters specific for human neuroendocrine cells are listed herein in Table 1. Any one or more of such promoters may be used in the context disclosed herein.

In preferred embodiments, the target cells will be human pancreatic β-cells, in which instances the preferred promoter will be an insulin gene promoter. Where the use of insulin gene promoters is contemplated, the promoter may be a rat insulin gene promoter (RIP), as exemplified by RIP1 or RIP2, or the promoter may be a human insulin gene promoter (HIP). In other preferred embodiments, the target cells will be pituitary cells, wherein the preferred promoters will be a growth hormone gene promoter or a proopiomelanocortin (POMC) gene promoter.

In preferred embodiments of the invention, the promoter used to direct expression of the immortalizing genetic construct will be a promoter that has been modified to have increased transcriptional activity, *i.e.*, it will be an "enhanced promoter". This includes both promoters that have been changed in terms of their base sequence, and promoters that comprise multimerized promoter elements. In terms of expression in pancreatic β-cells, an enhanced promoter may comprise an enhanced insulin gene promoter, such as one that comprises multimerized RIP elements or multimerized HIP elements.

There is no requirement for the at least a first immortalizing genetic construct to be introduced into the target cell by any particular means. Therefore, any and all means of providing the at least a first immortalizing genetic construct are contemplated to fall within the present invention. Transfection methods such as calcium phosphate, liposomes, gene gun, electroporation and even direct injection are particularly contemplated. However, it will often be preferred to provide the at least a first immortalizing genetic construct to the target cell by means of infection with at least a first recombinant virus that comprises the construct to be delivered.

Where viral infection is contemplated, there is again no particular requirement for any one or more viruses to be used. Therefore, any and all viruses suitable for use in gene delivery fall within the present invention. In preferred embodiments, it is currently contemplated that retroviruses, adeno-associated viruses (AAV), lentiviruses or adenoviruses will be used. The currently most preferred embodiment concerns the use of recombinant adenovirus or recombinant retrovirus. The combined use of one or more viruses, particularly the use of adenovirus with retrovirus, two adenoviruses and AAV with adenovirus, is contemplated in certain aspects of this invention.

Thus in certain aspects of the invention, the human neuroendocrine cell is provided with at least a first immortalizing genetic construct by two rounds of infection with a recombinant virus that comprises the construct. The cell may be provided with the immortalizing genetic construct by a first infection with a recombinant virus that comprises the construct followed by a second infection with a distinct, recombinant virus that comprises the construct. In other aspects, the cell is provided with a first and a second immortalizing genetic construct by a first infection with a recombinant virus that comprises the first construct followed by a second infection with a recombinant virus that comprises the second construct, the first and second immortalizing genetic constructs comprising distinct immortalizing expression units.

In further embodiments, the human neuroendocrine cell is provided with a first immortalizing genetic construct by transfection, and is then provided with a second immortalizing genetic construct by infection with a recombinant virus that comprises the second immortalizing genetic construct. The first and second immortalizing genetic constructs may comprise the same immortalizing expression unit, or distinct immortalizing expression units.

Thus, in certain preferred embodiments of the present invention, the starting human neuroendocrine cell is a primary islet cell, and the primary islet cell is provided with an adenovirus comprising at least a first temperature sensitive large T-antigen (TsTAg) immortalizing construct under the control of a cytomegalovirus (CMV) or a RIP promoter, and then provided with a retrovirus comprising at least a first telomere catalytic subunit (TERT) immortalizing construct under the control of a RIP promoter. In other preferred embodiments, the starting human neuroendocrine cell is an insulinoma, and the insulinoma is provided with an adenovirus or a retrovirus comprising at least a first temperature sensitive large T-antigen (TsTAg) immortalizing construct under the control of a CMV or a RIP promoter, and then provided with a retrovirus comprising at least a first telomere catalytic subunit (TERT) immortalizing construct under the control of a RIP promoter.

In still further embodiments of the invention, there are provided compositions and methods for use in regulating the expression of the at least a first immortalizing genetic construct when supplied to the human neuroendocrine cell. One method of control is to use at least a first temperature-sensitive immortalizing genetic construct, such as a temperature-sensitive large T antigen gene. Other methods of effecting control are provided herein and include methods in which the expression of the immortalizing genetic construct is conditional on the addition of an exogenous compound or component, such as using a Lac repressor system in conjunction with exogenously added IPTG, using a tetracycline regulatory system, which is dependent upon the addition of exogenous tetracycline or one or more tetracycline derivatives, or using the ecdysone-inducible expression system, which is dependent upon the addition of an ecdysone receptor ligand, including, but not limited to, ecdysone or the synthetic analog muristerone A.

Control of the at least a first immortalizing genetic construct may also be effected by actually physically removing the at least a first immortalizing genetic construct from the immortal human neuroendocrine cell once immortalization has been achieved. Any molecular biological method by which to prepare and use a removable genetic construct may be used in these aspects of the invention. A currently preferred system is Cre/Lox, in which the immortalizing genetic construct is flanked by *loxP* sites that are recognized by the Cre protein. Another preferred method by which to separate the immortalizing construct from the resultant immortal cell is to use a recombination activating system in which the immortalizing genetic construct is flanked by recombination signal sequences (RSSs), which are recognized by proteins encoded by genes termed RAG 1 or RAG 2 (Recombination Activating Gene 1 and 2). Yet another preferred method for removal of the immortalizing construct is the incorporation of the immortalizing construct on a non-integrative or extrachromosomal (episomal) adenoviral vector. As the immortalized cells divide, the adenoviral vector is continually diluted out as it is not integrated into the genomic DNA of the cells, thus eventually removing the immortalizing construct from the immortalized cells through normal methods of cell propagation.

Although the methods of the invention may be used to prepare an immortal human neuroendocrine cell that maintains all physiological functions and responses, it is not required that the resultant cell maintain every such biological function normally expressed by the cell type. In particular, modification and re-engineering of the secretory responses of the immortal cells may be achieved using further recombinant technology. So long as an immortal cell has been generated that retains the necessary secretory pathway components, the increased secretion of at least a first endogenous secretory polypeptide may be effected by the provision of one or more recombinant genes, such as, *e.g.*, processing genes.

The resultant immortal cells of the invention may also be modified or re-engineered to change the pattern of secretion of one or more secretory proteins or polypeptides in response to one or more modulators, such as secretagogues that normally act to stimulate secretion by the neuroendocrine cells, or inhibitors that normally act to inhibit secretion by the neuroendocrine cells. In certain embodiments, the human neuroendocrine cell secretes insulin, GLP-1, growth hormone, amylin or leptin in response to glucose and the cell is engineered to modify the secretion of GLP-1, growth hormone, amylin or leptin in response to glucose. One particular example of this is re-engineering immortal human β-cells to be glucose-responsive or to modify the glucose-responsiveness of the immortal cells. This may be achieved by the provision of one or more genes selected from an insulin gene, a GLUT-2 gene or a glucokinase (hexokinase IV) gene. U.S. Patent No. 5,427,940 is incorporated herein by reference in its entirety for the purposes of describing re-engineering of cells to provide glucose-responsive, insulin-secreting cells.

In certain preferred aspects of the present invention, it will be desired to block endogenous protein production in the immortal human neuroendocrine cells. For example further glucose-responsive engineering steps contemplated for use with the immortal human β-cells of the invention include various methods for reducing hexokinase I and/or hexokinase II activity within the immortal cells. Although the blocking of protein production is discussed below in terms of hexokinase reduction, it will be understood that one or more of these techniques will be applicable to the reduction of the production of any desired protein.

The reduction of hexokinase I and/or hexokinase II activity may be achieved by any one or more of a variety of methods. For example, one may use antisense technology, in which an antisense RNA molecule that is complementary to and binds to a hexokinase gene or RNA transcript is provided to the cell, and/or knockout technology, which refers to the application of a targeted gene disruption protocol (U.S. Patent No. 5,427,940), such as homologous recombination, random insertion or genomic site directed mutagenesis. Further methods for hexokinase reduction are provided herein and include the use of ribozymes targeted to hexokinase sequences, the use of various compositions that are effective to displace hexokinase from the mitochondria, and/or the use of one or more components or enzymes that act to stimulate the production of trehalose-6-phosphate.

The present invention further provides various methods of using the resultant human neuroendocrine cells disclosed herein. As certain aspects of the cells concern human therapy, the invention also provides immortal human neuroendocrine cells that have the capacity to be passaged *in vivo*. In further related methods of the present invention, the immortal human neuroendocrine cells or populations of such cells are grown in contact with a solid support or comprised within a bioreactor. In other methods, the immortal cells have been formulated in a pharmaceutically acceptable medium or vehicle, and immortal cells or populations thereof have been encapsulated within a biocompatible coating or implantable device.

In preferred aspects of the invention, the neuroendocrine cells provided will be immortal human cells, or a population of immortal human neuroendocrine cells, that secrete insulin in response to glucose. Such cells may be comprised within an implantable device or encapsulated in a biocompatible coating intended for use in treating an animal, such as a human subject, having, suspected of having, or at risk for developing pre-clinical diabetes or overt diabetes.

Distinct molecular biological compositions are also provided by the present invention. An example of such is an isolated DNA segment that functions to promote gene expression of a heterologous gene when the gene is operatively positioned downstream from the DNA segment, wherein the DNA segment comprises multimerized RIP promoter elements and comprises the DNA sequence of SEQ ID NO: 11. In particular, recombinant adenoviral and retroviral vectors and recombinant infectious adenoviruses and retroviruses are provided hereby which are, or comprise, a vector comprising at least a first immortalizing genetic unit, preferably at least a first telomerase catalytic component (TERT), under the transcriptional control of neuroendocrine cell-specific promoter operative in human neuroendocrine cells, preferably, a promoter listed in Table 1, and most preferably a promoter operative in pancreatic β-cells or pituitary cells.

Still further compositions of the present invention are recombinant vectors and gene delivery vehicles or viruses comprising such vectors, wherein the vectors comprise at least a first immortalizing genetic unit, in preferred embodiments at least a first telomerase catalytic component (TERT), under the transcriptional control of an insulin gene promoter comprising multimerized RIP promoter elements, wherein the vector expresses the immortalizing genetic unit specifically in human pancreatic β-cells.

In still further embodiments, the present invention provides various methods of using the immortal human neuroendocrine cells disclosed herein. Exemplary methods include those methods for producing selected polypeptides, which methods generally comprise first culturing a population of human neuroendocrine cells that secrete at least a first selected polypeptide, wherein the cells comprise at least a first immortalizing genetic construct, in preferred aspects at least a first telomerase catalytic component (TERT), under the control of a promoter, preferably a promoter specific for the neuroendocrine cells, and subsequently collecting the at least a first selected polypeptide so secreted.

The present invention also provides methods for the therapeutic provision of one or more selected proteins or polypeptides to human subjects, or even to animals. These methods generally comprise administering to a subject or animal believed to be in need of the one or more selected polypeptide an effective amount of a population of human neuroendocrine cells that secrete the one or more selected polypeptide, wherein the cells comprise at least a first immortalizing genetic construct, in certain preferred aspects at least a first telomerase catalytic component (TERT), under the control of a promoter, preferably a promoter specific for the neuroendocrine cells. In other preferred aspects, the cells secrete at least a first selected human polypeptide or peptide. Naturally, in such embodiments, the cells provided will preferably be encapsulated within a biocompatible coating or within a selectively permeable device.

An example of the treatment methods of the present invention is the treatment of diabetes in diabetic human subjects or even animals known or suspected to have diabetes. These methods generally comprise providing glucose-responsive insulin secreting capability to such human subject or animal *via* the administration of a biologically effective amount of a population of human neuroendocrine cells that secrete insulin in response to glucose, the cells comprising at least a first immortalizing genetic construct, in preferred aspects at least a first telomerase catalytic component (TERT), under the control of a promoter, preferably a promoter specific for the neuroendocrine cells. Again, it will be understood that such a population of cells would generally be provided to the animal in the form of an encapsulated biocompatible device.

The compositions and methods of the present invention can be used in connection with subjects "in need of one of the products secreted by the cells", such as, *e.g.*, insulin, growth hormone, amylin and the like. The "in need of" criteria covers any and all situations in which the subject would receive one or more benefits from provision of the cells and secreted products. Therefore, "in need of" covers prophylactic needs and benefits and disease maintenance needs and benefits, as well as therapeutic benefits, generally marked by an alleviation of symptoms or complications and/or an improvement in one or more clinical markers of disease. The "prophylactic" needs include subjects that are at risk of developing a disease state that can be prevented or delayed by a treatment method of the invention; whereas "maintenance" needs include subjects that are at risk of disease progression and/or acute or chronic complications thereof, which can be prevented or slowed by a treatment method of the invention.

In certain aspects of the present invention, the secretagogue responsive immortalized human neuroendocrine cells are advantageously used to deliver a physiologically relevant protein product in response to a physiologically relevant stimuli. The instant immortalized human neuroendocrine cells that secrete insulin in response to glucose are useful not only in diabetic insulin therapy, but also find use in reversing hyperglycemia, restoring normoglycemia, yet avoiding life-threatening hypoglycemia, and such like. The types of patients that would benefit from the methods and compositions of the present invention include those with Type I or insulin dependent diabetes mellitus (IDDM), Type II or non-insulin dependent diabetes mellitus (NIDDM), and gestational diabetes (women who either develop diabetic symptoms or whose pre-existing diabetic condition worsens incident to pregnancy).

In certain aspects of the invention, the insulin-secreting cells and methods can be used to provide a relatively constant supply of insulin to a patient in need of a constant insulin supply, the amount of which can be controlled simply by the number of immortalized human neuroendocrine cells administered. Therefore, in these aspects of the present invention, the glucose-responsiveness of the cells is not critical. For example, the claimed methods and compositions can be used in intensive insulin therapy. Intensive insulin therapy lowers the risk of complications of diabetes, such as diabetic retinopathy and nephropathy. Prior to the present disclosure, intensive insulin delivery was achieved simply using an external insulin pump or repeated injections, which cannot be regulated in response to changes in blood glucose. Thus the immortalized human neuroendocrine cells of the present invention can be similarly used to control glucose levels.

The clinical applicability and effectiveness of using the insulin-producing immortalized cells of the present invention, whether glucose-responsive or constitutively secreting, can also be readily monitored by determining the levels of one or more markers indicative of clinical benefit, for example microalbuminuria, albuminuria and/or glycated hemoglobin (HbA1c). Lowering of glycated hemoglobin towards the normal range is a much desired clinical goal for intensive insulin therapy and for reducing diabetic complications. Prior to the present disclosure, achieving normalized glycated hemoglobin levels has been difficult to achieve with conventional insulin therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. β-cell signal transduction and integration. Multiple signaling pathways are involved in the regulation of insulin secretion. Insulin secretion is regulated by effectors including metabolic fuels and hormones, and is subject to regulation as well by synthetic compounds. The various modulators of secretion exert effects *via* specific cell surface receptors (for example GLP-1, PP, IBMX, clonidine, epinephrine, somatostatin and carbachol), metabolic pathways (for example glucose, amino acids, FFA and IBMX), and ion fluxes (for example diazoxide, sulfonyl ureas, prandin, arginine and FFA). Most changes in secretion are mediated through changes in intracellular calcium.

FIG. 2A and FIG. 2B. Regulated secretion from engineered human cell lines. **FIG. 2A.** Fold insulin secretion (vertical axis) from βG 498/20 was measured in a two hour static incubation assay at basal conditions (0 mM; 1) or stimulated conditions: 10 mM glucose (2);

100 μM IBMX (3), 10 mM glucose + 100 μM IBMX (4); 100 μM carbachol (5); 100 μM carbachol + 10 mM glucose (6); 10 nM PMA (7); 10 nM PMA + 10 mM glucose (8); RPMI Medium + 100 μM diazoxide + BSA (9); or a stimulatory cocktail (RPMI medium supplemented with 10 mM glucose; BSA; 10 mM each arginine, leucine, glutamine; 100 μM carbachol, and 100 μM IBMX; 10) (horizontal axis). **FIG. 2B.** Insulin secretion (ng insulin/10^6 cells/2 hours; vertical axis) under basal (open bars) and stimulated (hatched bars) conditions from cell line βG 498/45 (7) and engineered cell lines 793/15 (1), 793/28 (2), 794/11 (3), 794/47 (4), 796/13 (5) and 796/15 (6) derived from βG 498/45 (horizontal axis). Cell line βG 498/45 (created by transfection of βG H03 with a plasmid conferring resistance to neomycin and encoding human insulin) was engineered for increased levels of insulin expression by the introduction of a number of plasmids, all of which encoded human insulin but varied in the genes encoding antibiotic resistance. The 793, 794, and 796 cell lines are resistant to mycophenolic acid, puromycin, and hygromycin, respectively. The data show the presence of a regulated secretory pathway in the progenitor cell line (498/45) and the maintenance of this capacity through a second round of engineering (793, 794, and 796 cell lines). The increase in stimulated secretion over basal secretion ranges from about 6- to 15-fold among the various clones.

FIG. 3. Insulin secretion (ng/flask/hour; vertical axis) under basal (cross-hatched bars) and stimulated (open bars) conditions from cell line βG 498/45 (6) and engineered cell lines 707/55 (1), 707/63 (2), 707/76 (3), 707/94 (4) and 707/96 (5) derived from βG H04 (horizontal axis). Engineered βG H04 cells fail to secrete insulin from the regulated secretory pathway. Transgenic (CMV-insulin/SV40-Neo), clonal derivatives of βG H04 known to secrete human insulin were tested for the capacity to secrete human insulin from the regulated secretory pathway. There was no difference between basal conditions (HBBSS with no glucose) and stimulated conditions (HBBSS + 25 mM KCl + 2.5 mM Forskolin + 50 μM IBMX) in the βG H04 clones: 707/55, 707/63, 707/76, 707/94, and 707/96. In contrast, in the clonal line derived from βG H03, there was a robust response to the aforementioned secretagogue cocktail, with about a 5-fold difference between basal and stimulated secretion.

FIG. 4. Schematic representation of a 2-step method for creating human neuroendocrine cell lines. Primary tissues, such as human islets; or neuroendocrine tumors, such as insulinomas, can be induced to proliferate through transgenic expression of growth-promoting proteins. A preferred protocol for such engineering is to selectively direct gene expression with the use of tissue-specific promoters and to provide transgenes *via* infection with recombinant adenovirus. Following an induction of proliferation, the cell population of interest is subject to enhanced rates of immortalization *via* infection with recombinant retroviruses.

FIG 5. Total insulin release (insulin, ng/24 hours; vertical axis) from a human insulinoma over time (days; horizontal axis). A freshly excised human insulinoma, about 1 cm^3, was processed and initially plated into two tissue culture wells, 9.6 cm^2 each. The cells that survived were subsequently aliquoted into a variety of culture conditions. At the times indicated, tissue culture media samples were obtained from each of the cell samples, and insulin was measured by RIA. The insulin output from the different samples was summed to give total output.

FIG 6. Acute insulin secretion (insulin, ng/50 IEQ/90 min; vertical axis) from human islets in BetaGene Medium supplemented with various concentrations of glucose (mM; horizontal axis). Islets were cultured in BetaGene Medium with 3.9 mM (□), 7.8 mM (O) and 22 mM (Δ) glucose for about 2 weeks. Although lower glucose was less deleterious than the higher concentration, both resulted in impaired secretory response.

FIG 7. Maintenance of human islets in BetaGene Medium supplemented with 1% fetal bovine serum (FBS; 1), 3.5% FBS (2), 10% FBS (3) or 5% horse serum (ES; 4) (horizontal axis). The serum requirements of human islets were tested in long term (at least 2 months) cultures supplemented with various amounts of serum, 1%, 3.5%, or 10% FBS and 5% horse serum (ES). In an acute secretion experiment, insulin secretion (insulin, ng/well; vertical axis) from islets cultured in 10% FBS exhibited lower response to glucose (2.2 mM, open bar; 11 mM, cross-hatched bar) or to a stronger mixed secretagogue stimulus (Swiss secretory cocktail, hatched bar). The sustained insulin output from human islets with 1% FBS

supplementation (in BetaGene Medium) suggested that human islets may also secrete insulin and survive under serum-free conditions.

FIG. 8. Comparison of commonly used medias to BetaGene Medium in the maintenance of human islets. Islets were cultured for 2-3 months with BetaGene Medium (1), Medium 199 (2), alpha MEM (3), or CMRL (4) (horizontal axis), all with equivalent glucose, and 0.1% BSA. In four independent islet isolations the insulin output (average insulin output, ng/well/day; vertical axis) was the highest with islets cultured in BetaGene Medium. In contrast, CMRL performed the poorest, essentially with no islet survival past 2 months with all 4 isolations studied.

FIG. 9. Long-term culture of human islets in BetaGene Medium restores and maintains glucose-stimulated insulin secretion. The capacity of BetaGene Medium to sustain the dose-responsive nature of the insulin secretory response (insulin, ng/50 IEQ/90 min; vertical axis) was evaluated with continuous cultures. Human islets were stimulated with varied glucose concentrations (mM; horizontal axis) at intervals to monitor secretory changes that may occur with time. A common finding was an initially poor response (shown at 1 week (O)), with increased function with time of culture in BetaGene Medium (6 weeks (☐) and 13 weeks (Δ)), and a maintained capability to secrete insulin in response to glucose for times >4 months.

FIG. 10A and FIG. 10B. Processing of proinsulin to mature insulin is enhanced by culturing human islets in BetaGene Medium. Insulin content was extracted from HI21 and fractionated by HPLC. Unprocessed insulin is retained longer on the HPLC column, while mature insulin elutes more rapidly. Insulin immunoreactivity (ng/ml) is shown on the vertical axis, and fraction number is shown on the horizontal axis. **FIG 10A.** Initially, 99% of the insulin was unprocessed insulin (peak centered about fraction 80; about 700 ng), with only 29 ng mature insulin/1000 IEQ. **FIG 10B.** The mature insulin content (peak centered about fraction 21) was increased 18-fold to 512 ng/1000 IEQ after 4 weeks of culture in BetaGene Medium; this represents >90% of the insulin content.

FIG 11A and FIG. 11B. Modified RIP activity in transiently transfected RIN cells. **FIG. 11A.** A schematic representation of the types of modified RIP promoters. **FIG. 11B.** Modified RIP promoter – human growth hormone (hGH) constructs (-205 RIP (1), -415RIP (2), FF3/-205RIP (3), FF6/-205RIP (4), -415RIP/FFE2/-205RIP (5), FFE3/-415RIP (6), FFE6/-415RIP (7); vertical axis) were transiently transfected into RIN cells. After 48 to 96 hours, hGH protein levels in the medium were determined by a radioimmunoassay (hGH levels relative to -415RIP (open bar); horizontal axis). The modified RIP promoters, FFE3/-415RIP and FFE6/-415RIP, were approximately 5-fold stronger than the RIP (-415RIP) promoter itself.

FIG 12A, FIG. 12B and FIG. 12C. Modified RIP activity in stably transfected RIN cells. The CMV promoter (5), RIP promoter (1), and several modified RIP promoters (FFE3/RIP (2), FFE6/RIP (3), FFE6/RIP/RIPint (4); vertical axis) were fused to insulin and were stably transfected into RIN cells. **FIG. 12A.** Insulin mRNA levels (shown relative to RIP mRNA level (open bar); horizontal axis) for each promoter construct were determined by Northern blot and quantitated with a phosphoimager. Cyclophilin mRNA levels were also determined by a phosphoimager as a control for Northern blot loading differences. **FIG. 12B.** Insulin protein levels secreted into the culture medium (insulin secretion relative to RIP (open bar); horizontal axis) were determined by a radioimmunoassay. **FIG. 12C.** Insulin protein levels within the cell (insulin content relative to RIP (open bar); horizontal axis) were determined by a radioimmunoassay after breaking open the cells by sonication. In all three cases, be it insulin mRNA levels, secreted insulin protein, or insulin protein content inside the cell, the modified RIP promoters were significantly stronger than the RIP promoter by itself. The FFE6 modified RIP promoters approach the activity of the very strong CMV promoter.

FIG. 13. Schematic representation of the mitogenic signal pathways in β-cells. Mitogenic pathways are shown for insulin-like growth factor-1 (IGF-1) and for growth hormone (GH). The IGF-1/IGF-1 receptor complex can signal cell mitogenesis *via* two pathways but in β-cells it does so primarily through the IRS pathway. Mitogenic stimulation of β–cells by GH is through the JAK/STAT pathway.

FIG. 14. IGF-1 stimulation of β-cell growth in the presence of increasing glucose concentrations. Shown are INS1 cells with no IGF-1 (open bars) and INS1 cells with IGF-1 (10 nM; hatched bars) incubated at different glucose concentrations (glucose, mM; horizontal axis). As judged by [^{3}H]-thymidine incorporation (fold increase in [^{3}H]-thymidine incorporation above no glucose/no IGF-1 control; vertical axis), glucose alone can initiate INS1 cell growth in a dose-dependent manner reaching a maximum of approximately 10-fold at 18 mM glucose. The effect of glucose on INS1 cell growth is potentiated by IGF-1 reaching a maximum of INS1 cell growth at 15 mM glucose.

FIG. 15. Growth hormone stimulation of β-cell growth in the presence of increasing glucose concentrations. Shown are INS1 cells with no rGH (open bars) and INS1 cells with rGH (10 nM; hatched bars) incubated at different glucose concentrations (glucose, mM; horizontal axis). The fold increase in [^{3}H]-thymidine incorporation above no glucose/no rGH control is shown on the vertical axis. The action of rGH, like that of IGF-1, requires a background of glucose to exert its effects. The rGH has little effect on cell growth until a threshold of 6 mM glucose and reaches a maximum at 15 mM glucose where there is an approximately 50-fold increase in [^{3}H]-thymidine incorporation over that at 0 mM glucose.

FIG 16. Additive effects of IGF-1 and rGH on β-cell growth. INS1 cells were incubated with no IGF-1 or rGH (□), 10 nM IGF-1 alone (■), 10 nM rGH alone (○), or both 10 nM IGF-1 and 10 nM rGH (●) at increasing glucose concentrations (glucose, mM; horizontal axis). The fold increase in [^{3}H]-thymidine incorporation above no glucose/no rGH control is shown on the vertical axis. As previously shown in FIG. 14 and FIG. 15, both IGF-1 and rGH potentiate the effect of glucose on INS1 cell growth to approximately the same degree. An additive effect on cell growth is observed when both growth factors are added to INS1 cells at the same time.

FIG 17A, FIG. 17B, FIG. 17C, FIG. 17D and FIG. 17E. Adenoviral overexpression of IRS-1, IRS-2, and SV40 large T-antigen in INS1 cells. Shown are uninfected INS1 cells (**FIG. 17A**) and INS1 cells were infected with either AdV-βGal (**FIG. 17B**), AdV-IRS-1 (**FIG. 17C**), AdV-IRS-2 (**FIG. 17D**), or AdV-large T-antigen (Tag; (**FIG. 17E**)) for 1 hour.

After 1 hour, the cells were washed and incubated another 24 hours. No IGF-1 (open bars) or IGF-1 (10 nM; cross-hatched bars) was added to the INS1 cells in the presence of either 3 mM or 15 mM glucose (glucose, mM; horizontal axis). The fold increase in [^{3}H]-thymidine incorporation above "zero" glucose control is shown on the vertical axis. Adenoviral-mediated overexpression of IRS-2 in INS1 cells in the presence of 10 nM IGF-1 and 15 mM glucose resulted in an approximately 200-fold increase in [^{3}H]-thymidine incorporation compared to uninfected cells without glucose. AdV-IRS-1 infected cells in the presence of 10 nM IGF-1 and 15 mM glucose showed no increase of [^{3}H]-thymidine incorporation over and above that for uninfected cells or cells infected with AdV-βGal in the presence of 10 nM IGF-1 and 15 mM glucose.

FIG 18. BetaGene Medium enhances growth of an engineered, human neuroendocrine cell line. The βG 785/5 cell line was derived from βG H04 cells which were derived and routinely cultured in RPMI with FBS. The growth rate (neutral red uptake, µg/well/hour; vertical axis) of βG 785/5 cells in BetaGene media with FBS (BG-FBS, ●) or SF (BG-SF, ○) and RPMI media with FBS (RPMI-FBS, ■) or SF (RPMI-SF, □), is shown. Time (days) is shown on the horizontal axis. Although cells grown in RPMI with FBS exhibited a longer lag phase, the growth of cells in BetaGene medium and RPMI with FBS was similar, all with doubling times of 2 days. However, cells in RPMI with SF essentially failed to grow, with an apparent doubling time of 26 ± 1 days.

FIG 19. BetaGene Medium enhances secretory function of an engineered, human neuroendocrine cell line (βG 785/5). Human growth hormone (hGH) output (µg/well/hour; vertical axis) of βG 785/5 cells in BetaGene media with FBS (BG-FBS, ●) or SF (BG-SF, ○) and RPMI media with FBS (RPMI-FBS, ■) or SF (RPMI-SF, □), is shown. Time (days) is shown on the horizontal axis. The human growth hormone (hGH) output of cells grown in BetaGene Medium with FBS was approximately 5 times greater than growth hormone output from cells in RPMI with FBS. Similarly, the hGH output of BetaGene Medium with SF was more than 5 times that of RPMI with SF. While BetaGene Medium supplemented with SF sustained hGH output equal to that of RPMI with FBS, it was not sufficient to support the same secretory function as BetaGene Medium with FBS.

FIG 20. BetaGene Medium maintains secretory function of βG 18/3E1 cell line. The insulin secretory function (insulin, μg/well/day; vertical axis) of βG 18/3E1 cells cultured in BetaGene Medium with SF (□) and with 0.5% FBS (Δ), 1% FBS (▲), 2% FBS (O) or 5% FBS (●) is shown. Days of culture is shown on the horizontal axis. The insulin secretory function of βG 18/3E1 cells was maintained when cells were cultured in BetaGene Medium supplemented with 5%, 2%, or 1% FBS. There was an impairment of secretory function with cells supplemented with 0.5% FBS or SF during the plateau phase of growth (about day 8 to 9 of culture). The secretory impairment at plateau phase under these conditions may be due to decreased biosynthesis or processing of insulin rather than an impairment of secretion.

FIG 21. Growth in BetaGene Medium maintains regulated secretion from the βG 18/E1 cell line. βG 18/3E1 cells were grown and maintained at plateau phase for 4 days in BetaGene Medium supplemented with SF + minerals (1), SF + minerals and amino acids (2), SF + amino acids (3), or 2% FBS (4) (horizontal axis). Basal (open bars) and stimulated (hatched bars) insulin secretion (ng/well/hour; vertical axis) in response to a secretagogue cocktail is shown for various SF- and 2% FBS- supplemented cultures in BetaGene Medium. This demonstrates that the capability of the regulated secretory pathway has been maintained, only the absolute output has been affected in both unstimulated and stimulated states, while the fold response is maintained.

FIG 22. BetaGene Medium enhances production of GLP-1 from an engineered, rodent neuroendocrine cell line. The capability of BetaGene medium to sustain processing and secretion of a peptide that yields proteolytically cleaved and amidated products was evaluated by measuring GLP-1 (amidated and non-amidated) production (GLP-1 immunoreactivity, pg/flask; vertical axis). βG 191/26 cells were plated in T25 flasks with BetaGene Medium and then the medium was switched to RPMI (1), RPMI with 75 μM ascorbate (2), or BetaGene Medium (3) (horizontal axis), all with 2% FBS. Both the total GLP-1 (open bars) and the amidated GLP-1 (hatched bars) output/day of cells in BetaGene Medium was essentially double that of cells in RPMI.

FIG 23. Ascorbate-2-phosphate supplemented media enhances insulin production of an engineered human neuroendocrine cell line. A suspension culture of βG 498/45 cells (PD33) were plated in varying concentrations (ascorbate concentration, mM; horizontal axis) of ascorbic acid (○) or A-2-P (Δ). Samples were collected for insulin assay (insulin, ng/well/day; vertical axis) and medium changed after 2 and 5 days of culture. In the initial 2 days of culture ascorbate altered insulin output by reducing insulin about 20%, only at the highest concentration. In the final 3 days cells, high concentrations of ascorbate were cytotoxic, while about 400 μM concentrations of both ascorbate and A-2-P enhanced insulin secretion. The highest concentration of A-2-P did not inhibit insulin output.

FIG 24. Media supplementation with ascorbate-2-phosphate can effect increased amidation activity with cultured cells. Production of amidated (hatched bars) and nonamidated (open bars) GLP-1 was determined by immunoassay (GLP-1 immunoreactivity, ng/100 μl assayed; vertical axis) of secreted cell products from cells cultured 1 day in RPMI medium (with 2% FBS) supplemented with varying concentrations of A-2-P (μM; horizontal axis). The dose-response shows half-maximal and maximal amidation activity with about 1 and 10-100 μM of A-2-P. The amount of amidated GLP-1 plateaued from 25-1000 μM. Concentrations of 10 mM consistently (4 separate experiments) resulted in slight decreases in amidated GLP-1, with a similar tendency to reduce non-amidated GLP-1 output. Supplementation with A-2-P results in a decrease in non-amidated GLP-1, such that amidated/non-amidated exceeds 100%. Maximal output of amidated GLP-1 with this cell line is about 12 pmol/million cells/day, representing a 5-fold increase over 0 μM A-2-P. This result demonstrates that supplementation with A-2-P can effect increased amidation activity in cultured cells.

FIG 25. Optimal copper concentration for PAM Activity. βG 191/26 cell monolayers in T25 flasks were changed to RPMI medium ± copper, or BetaGene Medium ± additional copper (the latter medium contains 5 nM copper). Shown on the horizontal axis are RPMI without $CuSO_4$ (1), RPMI with 0.5 μM $CuSO_4$ (2), BG without additional $CuSO_4$ (3), BG with 0.5 μM $CuSO_4$ (4), BG with 0.25 μM $CuSO_4$ (5), and BG with 1 μM $CuSO_4$ (6). Medium samples were collected after 24 h and the GLP-1 species (non-amidated (open bars)

and amidated (hatched bars)) were separated and quantified by HPLC (amount of GLP-1, ng/100 μl assayed; vertical axis). The results show that supplementing RPMI (which has no copper in its formulation) increases the output of amidated GLP-1. Further supplementation of BetaGene medium with copper to 250 and 500 nM does not increase amidated GLP-1, whereas 1 μM copper tends to decrease amidated GLP-1. These results indicate that 5 nM copper is adequate for PAM activity in cultured neuroendocrine cells.

FIG 26. Lack of Cytotoxic Effect of ascorbate-2-phosphate on Primary Human Islets. Human islets encapsulated in alginate beads were set up in 24 well plates with about 50 islet equivalents/well and cultured in BetaGene Medium with or without added A-2-P and copper. Control cultures 1 (□) and 2 (Δ), pretreatment (O), 0.5 mM ascorbate (●) and 2 mM ascorbate (■) are shown. Secretory function and glucose-sensing was determined by incubating the islets with different concentrations of glucose (from 2.2 mM to 22 mM; horizontal axis) for 90 minutes, and determining insulin secretion (insulin, ng/well/1.5 hr; vertical axis). This glucose dose-response test was performed immediately before adding ascorbate to the cultures and at 2 week intervals. In the first 2 weeks 500 μM A-2-P, and 1 μM copper was supplemented. In the second 2 weeks ascorbate was increased to 2 mM, copper was kept at 1 μM. A-2-P did not impair function as indicated by sensing of glucose, and the maintenance of maximal insulin secretion indicates that there is minimal toxicity of A-2-P for these culture times.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

In the development of alternative therapeutic strategies.for treating diabetes (improved over insulin injection), the provision of cell-based replacement therapies is particularly desirable. However, it is not currently clear whether successful cell-based product delivery, particularly that sanctioned by the FDA, will be best achieved with a xenograft, *e.g.*, with rodent cell lines, or using an allograft based upon human cell lines.

Human allograft development for insulin treatment is currently hindered by the lack of human cell lines that secrete insulin in a regulated fashion for a sustained period of time.

Likewise, an overview of other human diseases that result from absent or malfunctioning neuroendocrine cells of different types also reveals that the potential of cell-based systems for delivering biologically active peptides is currently hampered by the lack of human cells exhibiting the appropriate properties.

It is perhaps not surprising that various groups have attempted to develop human neuroendocrine cells, particularly human insulin-secreting cells and cell lines, for many years. Although many methods for achieving stable human neuroendocrine cells have been contemplated and described, the available approaches have all failed to achieve immortal, stable human neuroendocrine cells that maintain an effective or therapeutically relevant level of regulated peptide or polypeptide secretion over a significant period of time.

One strategy for generating human neuroendocrine cells concentrated on the generation of a "window" of human islet growth by manipulation of matrix and culture conditions. However, while it was reported that human islet cells could be stimulated to proliferate by growth on a 'biological matrix' produced by certain bladder carcinoma cell lines, and that this growth window could be enhanced by inclusion of hepatocyte growth factor/scatter factor (HGF/SF) (Hayek *et al.*, 1995; PCT application, WO 95/29989; U.S. Patent 5,116,753), the proliferation was later shown to be associated with duct cells present in the islet preparation, rather than the islet cells themselves (Lefebvre *et al.*, 1998).

Thus, the studies reported in Hayek *et al.* (1995), Beattie *et al.* (1991), WO 95/29989, and U.S. Patent 5,116,753 did not demonstrate generation of immortalized, transformed stable cell lines producing insulin, and were at best limited to short term studies of 5 to 14 days. Insulin content/µg DNA was shown to drop rapidly over the course of time (WO 95/29989), which is consistent with the lack of islet cell proliferation. Accordingly, the results were, at best, limited to defining conditions that allow some short term tissue culture of β-cells with little utility for generating a stable source of insulin-producing cells.

The manipulation of human islet cells during a transient proliferative phase induced by culture and/or matrix conditions has been described. These methods concern the use of retroviruses carrying oncogenes to transform the target cells *in vitro* (Levine *et al.* 1995). Since retroviral vectors can be stably integrated in growing cells, the reasoning appears to have been that human β-cell lines could be produced using this methodology.

This retroviral-oncogene approach has yet to yield stable cells derived from β-cells. The cell line established in Levine *et al.* (1995) secreted very small amounts of insulin in early passages (3 to 4 ng/million cells/24 hours) when stimulated by nicotinamide. Insulin secretion was also not stable, falling to undetectable amounts in later passages of the cell line. Furthermore, the H-*ras* of Levine *et al.* (1995) has separately been reported to induce β-cell destruction and diabetes in transgenic mice that express H-*ras* from the rat insulin II gene promoter (Efrat and Hanahan, 1989).

Another approach proposed for use in preparing an insulin-producing cell line is described in Neisor *et al.* (1979), and generally concerns the use of a temperature-sensitive Rous sarcoma or SV40 virus. This method is again limited by the lack of demonstration of stable cells derived from β-cells. Cell lines generated in Neisor *et al.* (1979) had very low insulin content (less than 1 ng/million cells, compared to normal islets which contain approximately 5 μg of insulin/million cells), and levels that quickly fell to undetectable levels after the third subculture.

U.S. Patent No. 4,332,893 reports to describe the generation of transformed, insulin-producing cell lines. However, the techniques described in this patent are not suitable for use with human neuroendocrine cells. The transformation methods rely on providing to an insulin-producing β-cell a temperature-sensitive strain of Rous sarcoma virus that contains a temperature sensitive lesion in the viral transforming sarc (*src*) gene.

However, U.S. Patent No. 4,332,893 itself states that these methods are not suitable for preparing human β-cells for use in the treatment of patients with diabetes. For example, it is stated that most temperature sensitive mutants of Rous sarcoma virus are "leaky," which means that the mutant gene product is not completely inactivated at the non-permissive temperature. In light of the evident problems in terms of safety risks that would result from the leakiness of cells administered to a diabetic patient, U.S. Patent 4,332,893 states that human pancreatic cells should not be transformed according to the methods described therein.

In preference to using human cells, U.S. Patent 4,332,893 teaches that cells designed for *in vivo* uses in human patients should be either bovine or porcine cells. The reasoning is that if the conditionally transformed bovine or porcine cells become leaky and proliferate, they are likely to rupture any semipermeable membrane within which they have been housed, thus releasing the transplanted cells into the human host. The use of the xenograftic cells will then cause the host patient to initiate a significant immune response which will lead to the rejection of the foreign cells, which the authors propose would take place before any oncogenic potential of the cells is realized.

A further significant limitation of the technology described in U.S. Patent No. 4,332,893 is that the transformation process described relies solely on the provision of a whole virus. The transformation events are thus reliant on the viral promoters and proteins produced during the infection. It will thus be seen that this method does not impart any cell-specificity to the transformation process and relies entirely on the initial selection of β-cells from a pancreas to produce a homogenous population of such cells.

It is well known in the art that the production of a homogenous population of β-cells from a pancreas and the culture and maintenance of such cells is difficult to practice without other, more hardy cells in the extracted population proliferating and outgrowing the β-cells. Therefore, in practicing the proposed methods of U.S. Patent No. 4,332,893 it seems unlikely that a homogenous population of β-cells would be obtainable, and it seems even more unlikely that contacting the initial population of cells would lead to the selective transformation only of β-cells within what must be supposed to be a heterogeneous population of cells.

In contrast, as will be seen below, the present invention provides many approaches to the selection and transformation only of β-cells, thus overcoming the difficulties not addressed by U.S. Patent 4,332,893, associated with the realization that a completely homogenous population of β-cells may not be purifiable.

The possibility of producing insulin-producing cell lines by cell fusion techniques are described in U.S. Patent 4,195,125 and PCT application WO 87/05929. Cells described in U.S. Patent 4,195,125 again secrete very low levels of insulin (2 to 3 ng/million cells/24 hours) and stability of these cell lines is not demonstrated. PCT application WO 87/05929 demonstrates generation of human liver cell lines but not of human β-cell lines.

A number of insulinoma cell lines have been established by expression of SV40 large T antigen in β-cells of transgenic animals under control of the rat insulin promoter/enhancer. Early lines produced by this method, such as βTC-1 and βTC-3, were shown to be glucose responsive, but with maximal secretion occurring at subphysiological glucose levels (Efrat *et al.*, 1988). In one cell line studied, the insulin secretion from βTC1 cells decreased by a factor of 10 between passage number 50 and passage number 63. Moreover, as a general trend observed for these cell lines, both the insulin content and insulin secretion decreased from passage 10 to passage 63.

More recently, two lines designated MIN6 and MIN7, which have different phenotypes and different insulin secretory profiles despite being generated with the identical insulin promoter-SV40 large T antigen construct, have been described (Miyazaki *et al.*, 1990). The MIN6 line shows a 10-fold increase in insulin secretion when glucose concentrations are raised from 5 to 25 mM, while the MIN7 line was found to be unresponsive to glucose. The MIN6 line was found to express high levels of the GLUT-2 glucose transporter and low levels of GLUT-1, while the MIN7 line expressed GLUT-1 and virtually no GLUT-2 (Miyazaki *et al.*, 1990).

Praz *et al.* (1983) reported that the cellular content of insulin in the RINm5F rat insulinoma cell line was only about 1% of the insulin content of native rat β cells. These authors also reported that there was no stimulation of insulin release even when glucose was increased from 2.8 to 33.4 mM. Overall, these findings were proposed to reflect a relatively specific impairment in glucose handling by the RIN cells. The foregoing findings were validated in later studies. For example, Giroix *et al.* (1985) reported that there was no significant difference in insulin output from RIN cells when the glucose concentration was raised from 2.8 to 16.7 mM. RIN cells were said to display severe abnormalities in the metabolism and secretory response to glucose. It was concluded that regulatory mechanisms distal to glucose phosphorylation were important in the control of glucose metabolism in insulin-producing cells.

Valverde *et al.* (1988) also reported that the RINm5F cell line displays a poor secretory response to glucose. These authors suggest that the tumoral RIN cells have a severe perturbation in their capacity to store proinsulin and insulin. In fact, a dramatic loss of insulin content is shown over a 12 month period, wherein the insulin content falls from 10.42 ng to 0.16 ng per million cells. It was also shown that the RIN cells contained a large fraction of proinsulin rather than properly processed insulin, which is at variance with the situation in normal islet cells and the recombinant cells of the present invention.

Clark *et al.* (1990) showed that the insulin content of unengineered RIN cells falls dramatically with passage. More importantly, these extensive studies on the RIN-38 cell line show that the ability of unengineered RIN cells to secrete insulin in response to glucose is abolished in cells with high passage number. Glucose-responsiveness is lost between passages 30 and 60, such that glucose-induced insulin secretion is completely absent at higher passage number. The instability is striking when comparing the response of cells at passage 22 and passage 78, where the higher passage cells are clearly non-glucose-responsive.

A separate rodent cell line derived by the transgenic T-antigen approach termed βTC-6, which, like MIN6, performs glucose stimulated insulin secretion with a concentration dependence similar to islets, and retains GLUT-2 and glucokinase expression in lieu of hexokinase and GLUT-1, has been reported (Efrat *et al.*, 1993). However, growth of these cells in culture results in a left shift in the glucose dose-response curve, with no apparent change in the expression of GLUT-2 and GLUT-1, a small increase in glucokinase activity, and a 6-fold increase in hexokinase. Very recently, the same group has used soft agar cloning of βTC-6 cells to establish a cell line (βTC6-F7) which retains glucose responsiveness at physiological glucose concentrations through at least 55 passages (Knaack *et al.*, 1994).

These cells express high levels of glucokinase and GLUT-2, low levels of hexokinase activity, and no detectable GLUT-1. The conclusion reached by these authors is that the original βTC-6 cell line was actually a heterogeneous population of cells, with the less differentiated cells in the population having a selective advantage in tissue culture over the more differentiated cells (Knaack *et al.*, 1994).

The ability of non-engineered HIT cells to secrete insulin in response to glucose has also been shown to progressively diminish with increasing passage number (Zhang *et al.*, 1989). These studies of HIT cells, derived from hamster pancreatic β-cells, led to the conclusion that the loss of insulin responsivity is a passage-dependent process that is serial rather than sporadic and global rather than glucose-specific. The Zhang studies further show that one cannot rely on stability over a "moderate" passage number, as a loss of phenotype still occurs later in such non-engineered clonal β-cells. In fact, a striking reduction in glucose-stimulated insulin secretion is shown, wherein the moderate response at passage 70 is markedly reduced by passages 75 and 81, and effectively absent in passages 83, 90 and 93.

Using yet another non-engineered β-cell line, mouse NIT-1 cells, loss of function has been reported at even lower passage numbers (Hamaguchi *et al.*, 1991). In common with RIN and βTC cells, NIT-1 cells also exhibit a decreased sensitivity to glucose as a function of time in culture, but this is evident as early as passage 16. It was shown that the glucose-stimulated insulin secretion is already lost by passage 16, with passage 19 showing a further decline in function.

Other insulinoma cell lines known as bHC have been isolated from hyperblastic β-cells of T antigen transgenic mice (Radvanyi *et al.*, 1993). These cells display well-differentiated phenotype but grow very slowly.

The studies described above demonstrate that transgenic expression of SV40 T antigen can be used to produce rodent islet β-cell lines, of which a small percentage appear to retain a well-differentiated phenotype. Irrespective of whether the resultant rodent cells prove to have the desired stability and/or properties, it will be understood that such a transgenic animal approach simply cannot be applied to the procurement of human cell lines. Furthermore, whether the cell lines described to date will remain differentiated for periods of sufficient duration to allow their use in hormone replacement therapy is still an open issue, as acknowledged by the groups from which they came (Efrat *et al.*, 1995). The foregoing animal studies also do not address the utility of T antigen for transformation of primary human neuroendocrine cells in culture.

Isolated reports have suggested that human islet cell lines could perhaps be generated by providing the cell with an oncogene under the control of a heterologous, or tissue-specific promoter (PCT application, WO 91/09939; Soldevila *et al.* 1991). However, prior studies in which human islet cells were provided, for instance, by electroporation with the SV40 large T antigen under the control of a viral promoter failed to generate any stable insulin-secreting cells (WO 91/09939; Soldevila *et al.* 1991). Initial levels of insulin production in Soldevila *et al.* (1991) were low (3 to 4 ng/million cells/ 24 hours) and in both references, insulin secretion fell to undetectable levels as the cell lines were cultured.

The disappointing results in WO 91/09939 and Soldevila *et al.* (1991) indicate that, in contrast to the initial speculation, the provision of a transforming, oncogene-like element to a population of cells derived from human islets did not result in the generation of stable β-cells.

Although certain other cell types have proven to be more easily manipulated, it is generally recognized in the art that human cells are difficult to immortalize (Shay *et al.* 1991a). Unfortunately, the few reports concerning the transformation of primary human cells with oncogenes, such as by using recombinant adenovirus expressing SV40 large T antigen (TAG), is of little relevance to the generation of stable human neuroendocrine cells. For example, although adenovirus expressing TAG has been used in connection with fibroblasts (Van Doren *et al.*, 1984; Van Doren and Gluzman, 1984), bone marrow stromal cells (Aizawa *et al.*, 1991), corneal epithelial cells (Araki-Sasaki *et al.*, 1995), bronchial epithelial cells (Reddel *et al.*, 1988) and esophageal epithelial cells (Inokuchi *et al.*, 1995), tissue culture conditions that allow vigorous mitotic growth have been established for each of these cell types, making transformation more likely, particularly using retroviruses. Furthermore, TAG expression has not been demonstrated in β-cells, and many neuroendocrine cell types, including β-cells, have limited capacity for cell division (Finegood *et al.*, 1995), and often lose their differentiated phenotype in tissue culture.

The engineered cells of U.S. Patent 5,427,940 make cell-based insulin replacement therapy for IDDM a realistic goal. To date, however, the absence of human neuroendocrine cell lines that retain a regulated pathway of hormone secretion has precluded the application of these methods to human cells, and efforts have thus been focused on various rodent cell lines (Newgard, 1994). While the description of genetic engineering methods for conferring glucose-stimulated insulin secretion in previously unresponsive rodent neuroendocrine cells is an important advance, it is as yet unknown whether rodent cell lines engineered in this fashion will be optimal in therapeutic terms or whether that will suffer from a certain degree of immune attack upon transplantation into human patients.

The procurement of an immortalized human neuroendocrine cell line would be a major advantage in that cells transplanted within a species (allograft) are generally less susceptible to immunological destruction than cells transplanted across species (xenograft). Although xenograft tissue, including recombinantly engineered cells generated by the methods of U.S. Patent No. 5,427,940, are generally intended for use in transplantation into diabetic animals and patients in the context of a selectively permeable capsule or device, the use of tissue from other species can still result in an immunological reaction.

As the administered cells themselves would be encapsulated within the biocompatible device, it will be understood that the immunological reactions associated with such a therapy will not generally include a significant antibody-mediated immune response. However, in that the administered cells would generally release one or more molecules, such as peptides, proteins, cytokines and the like, which would be small enough to diffuse through the semipermeable membrane, these released factors would be capable of initiating an immune response in the host animal or patient. In this scenario, cells of the immune system would be attracted to the area of implantation, and would be activated upon contact with the released foreign molecules, and would in turn produce various immunological effector molecules, such as cytokines and biological mediators such as TNF (tumor necrosis factor). Various of these smaller immunologically active species would also be capable of diffusing through the semipermeable membrane and would be able to attack or even destroy the cells encapsulated within the biocompatible device. It is in this manner that xenografts are less preferred than allografts.

In that the present invention provides for the generation of immortalized human neuroendocrine cells, these cells could be used in the form of an allograft for the treatment of diabetes in human patients. It is an advantage of the invention that the generation of stable human cells is complimentary to the technology of U.S. Patent No. 5,427,940, allowing the glucose-sensing engineering methods disclosed therein to be applied to the stable human cell lines generated by the present invention. It is contemplated that the combination of such approaches will produce cell lines that have high therapeutic relevance. However, prior to the present invention, such an approach could not be pursued due to the lack of stable human neuroendocrine cells.

The present inventors reasoned that previous methods to create an immortalized human neuroendocrine cell line, such as a human β-cell line, have failed due to the interplay among several potentially rate-limiting factors, particularly the use of methods that result in non-selective and/or uncontrolled transformation. Phenotypically differentiated β-cells have an extremely low rate of cell replication (Pittenger *et al.*, 1995), therefore it is not feasible to use stable gene expression systems that rely on cell replication to incorporate the proteins for transformation.

With heterogeneous populations of cells such as islets, which are made up of several distinct cell types - fibroblasts, ductal cells, α-, β-, and δ-cells - the use of immortalization methods that result in non-selective transformation results in cell lines of indeterminate phenotype. Loss of differentiated phenotype results from use of immortalizing methods that result in uncontrolled transformation (Soldevila *et al.*, 1991).

Also, the inadequate numbers of primary human islets have limited these technological efforts. Furthermore, the limited availability of human islets for experimental manipulation has hampered efforts to create a phenotypically stable β-cell line.

In terms of transformation itself, the inventors reasoned that the inefficient delivery of DNA that encodes transforming proteins to the cells and/or the nucleus and the use of insufficient numbers of proteins that cooperatively interact to drive transformation have been significant limitations.

It will be understood that the goal in creating an immortalized human β-cell is to produce a transformed β-cell line capable of replicating at a rate resulting in population expansion in culture, and maintaining sustained insulin secretion. The prior art methods of human islet tissue culture have, at best, only been able to produce a population of β-cells whose number is static due to very low rates of proliferation, and whose ability to produce insulin in a regulated manner is severely impaired (Brendel *et al.*, 1994); β-cells that divide at an enhanced rate but have suboptimal insulin content and secretory response (Otonkoski *et al.*, 1993); or transformed cell lines that de-differentiate and quickly lose normal function with respect to insulin (Soldevila *et al.* 1991).

The failure to distinguish the foregoing three types of candidate β-cells from true stable, insulin-secreting cells can create, at times, a degree of confusion in the art as to the availability of stable human β-cells. However, any confusion that may result following periodic scientific publications regarding the properties of new human β-cells is generally resolved with the failure, or death, of the cells originally described and the marked absence of further studies concerning such cells.

Transformation of human cells is a rare event both *in vivo* as evidenced by tumorogenesis (reviewed by Hunter, 1991) and *in vitro* as demonstrated experimentally (Shay *et al.*, 1991a). It is estimated that a minimum four to six genetic lesions or perturbations are required for development of human tumors (Peto *et al.*, 1975). Analysis of human tumors often reveals multiple genetic changes both in growth promoting oncogenes and in growth limiting tumor suppressor genes (reviewed by Bishop, 1991). Though rodent cells are more easily transformed than human cells, it is not believed that a single event can lead to transformation.

The ability of DNA tumor viruses such as SV40 large TAG to transform rodent cells and some types of human cells is related to their multifunctional properties. SV40 large TAG binds to and inactivates both p53 and Retinoblastoma (RB), two growth suppressor genes (Shay *et al.*, 1991b). In addition, transformation by SV40 large TAG is accompanied by chromosomal aneuploidy, and presumably promotes the accumulation of genetic lesions in cellular genes that regulate growth (Shay *et al.*, 1993). Not only are a multiplicity of events a requirement for transformation, it has also become apparent that proteins which promote transformation do so by acting cooperatively to promote growth and inhibit cell death (Hunter, 1991).

Various cell types differ in both in the frequency at which transformation occurs and in the identity of genetic lesions that are required for transformation. In humans the majority of cancers can be attributed to tumors of the lung, colon, breast, prostate, and skin (Beardsley, 1994). Each of these diseases originate in epithelial cells, which in the normal context are constantly undergoing division for the purposes of replenishment within their respective organs (Shay *et al.*, 1991a). Most other forms of cancer are rare by comparison.

Several lines of evidence suggest that pancreatic β-cells may be more resistant to transformation than most other cell types. First, adult human differentiated β-cells have little or no capacity for cell division. Any replenishment of differentiated β-cells in adults is likely to come from growth and differentiation of progenitor cells (Vinik *et al.*, 1996). The inability of humans to renew the β-cell population is demonstrated *in vivo* by the irreversible nature of Type I diabetes, a disease characterized by the destruction of β-cells (Unger and Foster, 1992), and *in vitro* by the difficulties associated with the expansion of islet cultures (Hayek *et al.*, 1995).

Second, insulinomas, β-cell tumors, are extremely rare in the population (Comi *et al.*, 1993), and, like β-cells themselves, these tumors also appear to have limited capacity for cell division, in contrast to many other types of tumors. Insulinomas are typically very small (0.5 to 1 cm) in size, and very rarely (~1% of all cases) exhibit metastatic potential. As predicted, patients with this tumor type do not present with pain or occlusion; rather, the clinical symptom is hypoglycemia. It is believed that the vast majority of insulinomas are the result of β-cell hyperplasia, and very rarely does this hyperplasia progress to a truly cancerous phenotype of aggressive growth, tissue invasion, and distal metastasis. Third, as detailed above, numerous investigators have been unable to achieve the stable transformation of β-cells using technologies that have successfully been applied to other human cell types.

In addition to differing in frequency of transformation, various cell types also differ in the identity of factors required for transformation (reviewed by Bishop, 1991). This is evident from analysis of human tumors. For example, carcinoma of the breast is often associated with aberrations in Neu and/or Myc; whereas colon cancers typically have mutations in k-Ras. Oncogenic mutations in the alpha subunit of Gs, a protein that regulates adenylate cyclase, have been found to be associated with pituitary tumors that secrete growth hormone (Landis *et al.*, 1989). However, Gs_α mutations are not present in a variety of other endocrine and neuroendocrine tumors (Lyons *et al.*, 1990).

Mutations that are associated with insulinomas are much less characterized and understood than those found in commonly occurring tumors. Two insulinoma-associated (IA) cDNAs have been found to be overexpressed, relative to normal islet tissue, in human and rodent insulinomas. One of these, designated IA-2, encodes a protein that is related to the protein tyrosine phosphatase family and is thought to be a cell-surface receptor. Of several human tumors and cell lines examined, expression of IA-2 is restricted to insulinomas and a glioblastoma cell line (Lan *et al.*, 1994). The other cDNA is designated IA-1 and is a member the zinc-finger transcription factor family. IA-1 also shows a restricted expression pattern, with a overexpression occurring mainly in neuroendocrine tumors (Goto *et al.*, 1992).

Additional clues to the genetic basis for β-cell transformation have come from the study of individuals with hereditary multiple endocrine neoplasia, type 1 (MEN1) (reviewed by Thakker, 1993). This disorder is characterized by the combined and increased occurrence of tumors of the parathyroid, pancreatic islet cell, and anterior pituitary. The gene linked to MEN1 maps to chromosome 11, band 11q13, and the corresponding gene has recently been cloned

(Chandrasekharappa *et al.*, 1997). Menin, the protein encoded by this gene, has been demonstrated to be a nuclear protein (Guru *et al.*, 1998), and to function in normal cells as a tumor suppressor, with most neuroendocrine tumors studied to date having a double mutation in MEN1. It has thus been proposed that the mutation associated with MEN1 functions in a restricted fashion in transformation, and may be uniquely involved in the development of endocrine tumors.

Furthermore, while cell lines from certain tumor types, particularly PCC, lung, thyroid and gastrointestinal, have been reported, this apparent success has not been translated to tumors of the β-cell or pituitary. Thus, after analyzing the foregoing phenomena and results from many diverse studies, the inventors reasoned that the successful generation of an immortalized human neuroendocrine cell, particularly an immortalized β-cell, will likely require the combination of both different techniques and diverse biological components, not previously suggested for combined use in human β-cell transformation.

Where transforming components are concerned, the inventors reasoned that improvements in the efficiency of DNA delivery for selective, stable gene expression of transforming proteins, and the expression of more than one protein simultaneously, which proteins cooperatively interact to drive transformation in a more controlled or refined manner, will overcome the problems that previously hindered the development of a phenotypically stable neuroendocrine cell line. The inventors envisioned that the cooperation of multiple proteins that regulate cellular growth, senescence, and apoptosis will be preferable.

Currently, there are over sixty known viral and cellular proteins that have been implicated in the transformation of normal cells (Schwechheimer and Cavenee, 1993, incorporated herein by reference). Oncogenes and tumor suppressors can alter cell growth by several modes of action including perturbations in extra- and intra-cellular signaling (Cross and Dexter, 1991; Cantley *et al.*, 1991; Baserga, 1994); direct effects on the cell cycle (Cordon-Cardo, 1995; Arnold, 1995; Kamb, 1995); inhibition of tumor suppressor genes and proteins (reviewed by Hale *et al.*, 1996; Marshall, 1991); and alterations in nuclear transcription (Hunter, 1991).

Recently, an additional protein has emerged as a potentially universal contributor to cellular transformation. Telomerase activity, defined as the lengthening of telomeres at the end of chromosomes, is expressed at relatively high levels in most human cancers that have been examined to date (Shay and Wright, 1996). The catalytic subunit of telomerase, referred to as TERT or EST2, has recently been isolated (Nakamura *et al.*, 1997; Meyerson *et al.*, 1997). Transfection of TERT into epithelial cells and fibroblasts has been shown to increase the normal cellular life-span (Bodnar *et al.*, 1998).

In preferred aspects of the invention, the immortalizing construct comprises the catalytic subunit or component of telomerase. The catalytic subunit of telomerase will not need to have full or complete activity to find utility in many aspects of the present invention. Thus, as used in the present invention, the catalytic subunit will have sufficient activity to extend the end of a chromosome by at least one telomeric repeat unit. The telomerase activity can be measured by any of the telomerase assays described in detail herein below. In any event, the catalytic subunit of telomerase will have sufficient activity to effect the immortalization of the particular human neuroendocrine starting cell utilized.

In seeing the various limitations of the prior art as a whole, the inventors have developed, for the first time, methods for the generation of immortalized human neuroendocrine cell lines, such as human β-cell lines, that exhibit sustained insulin secretion. The inventors reasoned that the generation of immortalized human neuroendocrine cells would, in fact, be achievable through the use of promoters, for example tissue-specific promoters, to direct expression of transforming genes, for example the catalytic subunit of telomerase, in specific target cells within a population of human cells, despite the lack of success reported by previous investigators employing related techniques. Accordingly, the invention provides immortalized cells of human neuroendocrine lineages that have maintained their differentiated phenotype and thus have a defined, regulated secretory pathway and are stable in *in vitro* culture. The present invention also provides methods for making and using such cells.

As detailed below, in certain preferred embodiments, the present invention concerns the use of tissue specific promoters that have been engineered to provide greater transcriptional activity. This is exemplified by the rat insulin promoters (modRIP) containing multimerized enhancer elements.

A currently preferred method of delivering the promoter-immortalizing construct, or preferably the tissue-specific promoter-telomerase catalytic subunit construct, to the target neuroendocrine cells is to use infection with recombinant adenoviruses. This results in a high efficiency of gene delivery to cells that are normally refractory to other methods of DNA transfection.

In further preferred embodiments, the invention contemplates the use of several transforming genes in combination, and in certain embodiments distinct viral vectors (*i.e.*, adenoviral and retroviral vectors) to deliver transforming genes in combination. In still further preferred embodiments, the transformation methods of the invention concern the surprising use of defined culture additives, such as growth factors, that further promote specific neuroendocrine cell growth in preference to that of other cell types, or toxins that specifically inhibit the growth of unwanted cell types.

The use of a combination of transforming genes and/or conditions that interact cooperatively to drive transformation is also preferred. As an example of this embodiment, human β-cells are provided with a hormone receptor expressed from a tissue-specific promoter. β-cells are then selectively stimulated to proliferate by the addition of the cognate hormone to defined media. With a dividing β-cell population, retroviral constructs are then used to efficiently deliver one or more transforming constructs to the genome.

In other preferred embodiments, the ability to control expression of the transforming gene is included, as exemplified by temperature-sensitive constructs and by ecdysone-inducible promoters or promoters containing lac operon sites, so transcription is regulatable.

I. Human Secretory Cells

A. Functional, Regulated Secretory Pathways

Secretory cells, especially neuroendocrine cells, have several endogenous functions that make them uniquely suited for production of a wide range of proteins, including secreted peptide hormones. These specialized functions are encompassed by the regulated secretory pathway. The regulated secretory pathway embodies the secretory granules of neuroendocrine cells which serve as the site of maturation and storage of a large class of peptide hormones with profound biological functions. Proper biological function of the peptides is due both to their secretion in a regulated and titratable manner as well as a complex set of post-translational modifications resulting in the final biologically active product. As a result, these cells can be used *in vitro* to produce large amounts of proteins, *in vitro* and/or *in vivo* as drug discovery/development tools, *in vivo* to supply therapeutic proteins, or *in vivo* to immunize hosts, for example, even in the production of monoclonal antibodies.

The generation of stable regulated secretory cells which synthesize proteins for either *in vitro* or *in vivo* applications will advantageously make use of many attributes of these cells. Regulated secretory cells present a natural bioreactor containing specialized enzymes involved in the processing and maturation of secreted proteins. These processing enzymes include endoproteases (Steiner *et al.*, 1992) and carboxypeptidases (Fricker, 1988) for the cleavage of prohormones to hormones, and PAM, an enzyme catalyzing the amidation of a number of peptide hormones (Eipper *et al.*, 1992). Similarly, maturation and folding of peptide hormones is performed in a controlled, stepwise manner with defined parameters including pH, calcium and redox states.

Complete processing requires sufficient levels of the processing enzymes as well as sufficient retention of the maturing peptides. In this way, physiological signals leading to the release of the contents of the secretory granules ensures release of fully processed, active proteins. This is important for both maximum production for *in vitro* purposes and for the possible use of cells for *in vivo* purposes.

All cells secrete proteins through a constitutive, non-regulated secretory pathway. A subset of cells are able to secrete proteins through a specialized regulated secretory pathway. Proteins destined for secretion by either mechanism are targeted to the endoplasmic reticulum and pass through the Golgi apparatus. Constitutively secreted proteins pass directly from the Golgi to the plasma membrane in vesicles, fusing and releasing the contents constitutively without the need for external stimuli. In cells with a regulated pathway, proteins leave the Golgi and concentrate in storage vesicles or secretory granules. Release of the proteins from secretory granules is regulated or modulated, requiring an external stimuli, or modulator. This external stimuli can either effect secretion or inhibit secretion. External stimuli that effect secretion, defined as a secretagogues, or those that inhibit secretion, defined as inhibitors, can vary depending on cell type, optimal concentration of the particular secretagogue or inhibitor, and dynamics of secretion. Proteins can be stored in secretory granules in their final processed form for long periods of time. In this way a large intracellular pool of mature secretory product exists which can be released quickly upon secretagogue stimulation.

A cell specialized for secreting proteins *via* a regulated pathway can also secrete proteins *via* the constitutive secretory pathway. Many cell types secrete proteins by the constitutive pathway with little or no secretion through a regulated pathway. As used herein, "secretory cell" defines cells specialized for regulated secretion, and excludes cells that are not specialized for regulated secretion. The regulated secretory pathway is found in secretory cell types such as endocrine, exocrine, neuronal, some gastrointestinal tract cells and other cells of the diffuse endocrine system.

The origin of the starting cells for use in the present invention thus include human tissues and tumors of neuroendocrine lineages that have a well defined regulated secretory pathway. Cells with defined conditions for culturing *ex vivo* with some replicative capacity are also preferred. Pancreatic β-cells and pituitary cells are preferred for use in the present invention, with β-cells being more preferred. Examples of such cells are shown in Table 1 below (Pearse and Takor, 1979; Nylen and Becker, 1995).

TABLE 1

Neuroendocrine Cell Types

Neuroendocrine cell	Endogenous hormone	Tissue specific promoter	Context specific promoter	Associated Tumors
Hypothalamic/pituitary cells				
Corticotropes	ACTH,LPH		POMC (V01510, K02406)	Corticotrope adenoma
Somatotropes	Growth Hormone	Growth Hormone (J03071, K00470)		GH producing adenoma
Melanotropes	alpha-MSH, endorphins		POMC (V01510, K02406)	
Lactotropes	Prolactin	Prolactin (X00368, L33865)		Prolactin adenomas
Thyrotropes	Thyroid-stimulating hormone	TSH (M23669, S70587)	α-glycoprotein (L05632)	Thyrotrope adenomas
Gonadotropes	Follicle Stimulating hormone	FSH (M16646), LH (X00264)	α-glycoprotein (L05632)	Gonadotrope adenoma
	Leuteinizing hormone		α-glycoprotein (L05632)	
Adrenal medulla	enkephalins, dynorphin NPY, bombesin	NPY (M14296)		

TABLE 1 - CONTINUED

	NPY, bombesin			
Thyroid C Cell	Calcitonin, CGRP, Somatostatin, Bombesin		Calcitonin (X15943), Somatostatin (J00306)	Thyroid carcinomas
Parathyroid Chief Cells	Parathyroid hormone	Parathyroid hormone (J00301)		parathyroid adenomas
Pulmonary neuroendocrine cells/ K cells	Calcitonin, Bombesin, CGRP, cholecystokinin, endothelin		Calcitonin (X15943)	Small Cell Lung Carcinoma
Gastric G cells	Gastrin, enkephalin		Gastrin (X00183)	Gastrinoma
Gastric D cells	Somatostatin		Somatostatin (J00306)	Somatostatinoma
Enteroneuroendocrine cell types				
L cells	Glucagon Family peptides Peptide YY	Peptide YY (L25648, D13897)	Glucagon (X03991)	Glucagonoma
D cells	Somatostatin		Somatostatin (J00306)	Somatostatinoma
S cells	Secretin	Secretin		

TABLE 1 - CONTINUED

G cells	Gastrin		Gastrin (X00183)	Gastrinoma
K cells	GIP	GIP (M31674)		
I cells	CCK	CCK (L29399, M15843, N00050)		Enteroneurocrinomas
N cells	Neurotensin	Neurotensin (S47339)		Neurotensinoma
H cells	VIP		VIP (M33027, M37460)	VIPomas
Enterochromafin cells	Motilin, Substance P, Substance K	Motilin (X15392, Y07505, M30277)	Substance P (M68906)	Carcinoid tumors
Pancreatic Cells				
β-cells	Insulin, amylin	Insulin (V00565), amylin (L08226)		Insulinoma
α-cells	Glucagon, Corticotropin releasing hormone	Glucagon (X03991), Corticotropin releasing hormone (X55962)	Glucagon (X03991)	Glucagonoma
PP cells	Pancreatic polypeptide	Pancreatic polypeptide (M11726)		PPoma
δ-cells	Somatostatin		Somatostatin (J00306)	Somatostatinoma
G cells	Gastrin		Gastrin (X00183)	Gastrinoma

TABLE 1 - CONTINUED

Carotid I cell	Substance P, enkephalin		Substance P (M68906)	Carcinoid tumors
Urigenital tract Merkel cells	Calcitonin, bombesin, VIP	VIP (M33027, M37460)	VIP (M33027, M37460)	VIPomas
syncytiotrophoblast cell	chorionic-gonadotropin	chorionic-gonadotropin (M13504)		Trophoblast tumors
Neurons				
Supraoptic and paraventricular nuclei	Vasopressin, oxytocin	Vasopressin (X62890), oxytocin (M11186)		
Sympathetic ganglion	VIP, enkephalin		VIP (M33027, M37460)	VIPomas
Paraganglia	enkephalin			

B. Secretagogue-Responsive Polypeptide Production

The term "regulated secretory pathway" means that the amount and/or rate of secretion of an endogenous polypeptide can be stimulated by external stimuli, commonly referred to as secretagogues. Secretagogues can be physiological in nature, *e.g.*, glucose, amino acids, or hormones, or pharmacological, *e.g.*, IBMX, forskolin, or sulfonylureas. Polypeptides destined for the regulated secretory pathway are stored in intracellular storage vesicles known as secretory granules.

C. Neuroendocrine Cell Sources

1. Fetal Cells

In certain aspects of the present invention, fetal cells are preferred for use as the starting human neuroendocrine cells. Human fetal organs, such as fetal pancreases at 18 to 24 gestational weeks, can be obtained through nonprofit organ procurement centers, with patient consent for tissue donation being obtained. Dissection of specific organs from the fetuses is often done at the procurement centers. Isolation of fetal pancreases and islets is performed by established techniques (Otonkoski *et al.*, 1993; incorporated herein by reference).

2. Cells from Primary Human Tissues

In other aspects of the present invention, cells from primary human tissues are preferred for use as the starting human neuroendocrine cells. Human organs can be obtained from autopsies through nonprofit organ procurement centers. High quality human islets are available, for example, from Dr. Camillo Ricordi of the University of Miami Medical Center, an islet transplant surgeon who supplies human islets to scientists throughout the United States. Automated methods for isolation of human pancreatic islets have been established (Ricordi *et al.*, 1988; incorporated herein by reference).

3. Cells from Resected Neuroendocrine Tumors

Explanted tumor samples from surgically resected tumors are another preferred starting material. More preferred are insulinomas and pituitary tumors. A preferred option as a starting material for achieving a human β-cell line is to use an insulinoma cell line from a resected human insulinoma tumor.

Two exemplary insulinomas have been reported (Gueli *et al.*, 1987; Cavallo *et al.*, 1992). In general, these cells are likely to have difficulty processing proinsulin to insulin (Comi and Gorden, 1995), and often express significant levels of other select hormones, such as glucagon and somatostatin.

The inventors obtained ten independent human insulinomas and maintained these samples in cultures for variable periods of time. Typically the tumor specimens display time-limited capacities for cellular proliferation and a progressive loss in insulin production. Prior to the present disclosure, no certifiable human β-cell lines had been established from resected insulinomas. This is in contrast to the success researchers have had in establishing human cell lines from other tumor types. For example, researchers at the National Cancer Institute have reported success rates of 35% in establishing cell lines from small cell lung carcinomas (Gazdar and Minna, 1996).

Several factors may underlie the inherent difficulty in establishing cell lines from human insulinomas, including inappropriate culture conditions, activation of tumor suppressor genes *in vitro*, and most likely, insufficient expression of genes involved in cellular immortalization. The clinical presentation and pathophysiology of insulinomas supports this latter possibility. Insulinomas are typically very small (0.5 to 1 cm) in size and, very rarely (~1% of all cases), exhibit metastatic potential. As predicted, patients with this tumor type do not present with pain or occlusion; rather, the clinical symptom is hypoglycemia. It is believed that the vast majority of insulinomas are the result of β-cell hyperplasia, and very rarely does this hyperplasia progress to a truly cancerous phenotype of aggressive growth, tissue invasion, and distal metastasis. The inventors reasoned that the same factors that tend to make insulinomas a relatively benign tumor type are the same factors that are rate-limiting in the establishment of cell lines *in vitro*.

Although none of the described human "insulinomas" actually have the properties required to be properly described as stable human β-cells, the techniques of the present invention are still suitable for use with such cell populations as starting materials in order to procure a pure population of stable, human insulin-producing cells from the mixture of cells currently available.

4. Cells of Human Neuroendocrine Cell Lines

It will be understood that tumor cell lines and insulinomas arising from explants of resected neuroendocrine tumors are not necessarily, by definition, stable cells; some such cells maintain a differentiated phenotype for two, four or about six months at the maximum. For example, the NCI has approximately 250 cell lines, about 100 of which are reported to possess some level of neuroendocrine properties. However, such cells are intended for use as starting materials in the present invention. Table 2 below is exemplary of these human neuroendocrine cell lines.

Table 2 describes the properties of certain exemplary cell lines that are contemplated as starting cells for use in certain aspects of the instant application.

TABLE 2

Human Cell Lines

βG ID	NCI/ATCC	Origin	NEprofile PEP$^{+/-}$	SELEC AB$^{S/R}$	TG$^{+/-}$	NuTum Grow$^{+/-}$	AlgBeads		GHT device	
							vitro	vivo	vitro	vivo
H01	H716/CCL-251	Colon	PAM+, SYN+, PC1/PC3+, PC2-, VIM+, CPE+	G^S, H^S, O^S	-	I^-, GH^-, G^-	-	NT		
H02	NA/CRL-1803	Thyroid	PAM+ , SYN+, PC1/PC3+ , PC2+ , VIM-, CPE+	G^R, H^R O^R			NT	NT		
H03	H810/CRL-5816	Lung	PAM+, SYN+, PC1/PC3+ , PC2+ , VIM-, CPE+	G^S, H^S, O^S, P^S, B^S, Hd^S, Mca^S, Z^s	NP^+, I^+, G^+, GH^+, G2+, CD, AR	+	+	+	+	
H04	H1299/CRL-5803	Lung	PAM+ , SYN-, PC1/PC3+, PC2-, VIM+, CPE-	G^S, H^S, O^S	I^+, CD+, G+/-		NT	NT		
H05	H378/CRL-5808	Lung	PAM+, SYN+, PC1/PC3(+), PC2+, VIM-, CPE+	G^R, H^R, O^R			NT	NT		
H06	H727/CRL-5815	Lung	PAM+, SYN+, PC1/PC3+, PC2-, VIM-, CPE+	G^R, H^R, O^S			NT	NT		
H07	H187/CRL-5804	Lung	PAM+, SYN+, PC1/PC3+, PC2-, VIM-, CPE+/-	G^S, H^S, O^S			NT	NT		

TABLE 2 - CONTINUED

H08	H1385/CRL-5867	Lung	PAM+, SYN-, PC1/PC3(+) , PC2+, VIM+, CPE+	G^S, H^R, O^R			NT	NT		
H09	H720/CRL-5838	Lung	PAM+, SYN+, PC2+, PC1/PC3+, VIM-, CPE+	G^S, H^S, O^S		-/+	+	NT		
H10	NA(A-549)/CCL-185	Lung, ES	PAM+, SYN, PC1/PC3(+) , PC2-, VIM+, CPE-NT	G^S, O^{NT}, H^{NT}	GH+		NT	NT		
H11	NA/HTB-9	Bladder	PAM+, SYN-, PC1/PC3-, PC2-, VIM+, CPE-	G^S, H^S, O^S	G-, I-, GH-		NT	NT		
H12	NA/(CM from Pozilli)	Insulinoma	PAM+, SYN+, PC1/PC3-, PC2-, VIM+, CPE-NT	G^S, H^S, O^S, P^S			NT	NT		
H13	NA/CRL-2139	Neuroect-odermal	PAM+, SYN-, PC1/PC3(+) , PC2-, VIM+, CPE+/-	G^S, H^S, O^S			NT	NT		
H14	H548/CCL-249	Colon	PAM-NT, SYN-NT, PC1/PC3-NT, PC2-NT, VIM-NT, CPE-NT	NT			NT	NT		
H15	H508/CCL-253	Cecum	PAM+, SYN+, PC1/PC3-, PC2-, VIM-, CPE-	G^S, H^S, O^S	NP^+		+	NT		

TABLE 2 - CONTINUED

H16	H1770/CRL-5893	Lung	PAM+, SYN , PC1/PC3 (+), PC2+, VIM-, CPE+	G^S, H^S, O^S			NT	NT		
H17	NA/CRL-5974	Gastric	PAM+, SYN+, PC1/PC3-, PC2- , VIM-, CPE-	G^S, H^S, O^S	NP^+		+	NT		
H18	H345/HTB-10	Lung	PAM+, SYN+, PC1/PC3+, PC2+, VIM-, CPE+	G^S, H^S, O^S	NP^+		+	NT		
H19	H510A/HTB-184	Lung	PAM+, SYN+, PC2+, PC1/PC3+, VIM-, CPE+	G^S, H^S, O^S	NP^-, I^-		-	NT		
H20	H460/HTB-177	Lung	PAM+, SYN-, PC1/PC3-, PC2-, VIM+, CPE-	G^R, H^R, O^R			NT	NT		
H21	NA/CRL-2195	Lung	PAM+, SYN+, PC1/PC3+, PC2+, VIM-, CPE+	G^S, H^S, O^S, Z^S			I-/+, G+	+	+	+
H22	H2098/Gazdar insulinoma	Insulinoma	PAM+, SYN+, PC1/PC3+, PC2+, VIM-, CPE+	G^S, H^S, O^S			NT	NT		
H23	NA/NA (BON)	Pancreatic Carcinoma	PAM+, SYN+, PC1/PC3+, PC2- VIM+, CPE+	G^S, H^S, O^S	NP^+, I^-, G^+		+	+	+	+

TABLE 2 - CONTINUED

H25	NA (HepG)/HB-8065	Hepatocytes	PAM+, SYN-, PC1/PC3-, PC2-, VIM-, CPE+/-	G^S, H^{NT}, O^{NT}	N^+		NT	NT		
293		Kidney	PAM+, SYN-, PC1/PC3-, PC2-, VIM+, CPE-	G^S, H^S, O^S	NP^+, I^+, G^+, GH^+, G2+		+	+	-	-

KEY: NCI, National Cancer Institute; ATCC, American Type Culture Collection; NE, neuroendocrine; PAM, peptidylglycine alpha-amidating monooxygenase; SYN, synaptophysin; PC, proconvertase; VIM, vimentin; AB, antibiotic; S/R, sensitive/resistant; G, G418; H, hygromycin; O, ouabain; P, puromycin; B, blasticidin; Hd, histidinol; Mca, mycophenolic acid; Z, Zeocin; TG, transgene expression +/-; NP, neomycin phosphotransferase; I, insulin; G, glucagon/glycentin; GH, growth hormone; NT, not tested

βG H03 cells are derived from a human non-small cell lung carcinoma (ATCC Number: CRL-5816). These cells have a neuroendocrine phenotype, and can be grown in a monolayer. This line was derived by Gazdar and associates from a lung tissue obtained from a patient prior to therapy. H03 cells as obtained from the ATCC are not able to synthesize the peptide neuromedin B (NMB) or the gastrin releasing peptide (GRP).

Other lung carcinoma cells include cells designated herein as βG H04, βG H05, βG H07, βG H09, βG H19, βG H20 and βG H21. These, as well as additional cells lines derived from other sources, are described in further detail herein below. These cell lines are only exemplary starting cells for use in the present application; given the teachings provided herein, one of ordinary skill in the art will be able to identify additional cells with characteristics that would make them appropriate for use in the present invention.

H01 cells (ATCC Number: CCL-251) also may be used in the present invention. These cells are human colorectal carcinoma cells having an epithelial morphology. These cells grow in floating aggregates of round cells. A characteristic that makes these cells useful in the context of the present invention is that they contain cytoplasmic dense core granules characteristic of endocrine secretion.

βG H02 cells are obtained from the ATCC (CRL-1803) are derived from a thyroid medullary carcinoma. Their morphology is epithelial and are known to produce high levels of calcitonin and carcinoembryonic antigen (CEA). Chromosomal analysis of the cell line and tumors induced in nude mice reveal an aneuploid human karyotype with several marker chromosomes.

βG H04 cells are obtained from the ATCC (CRL-5803) are lung carcinoma cells and have a neuroendocrine phenotype. The cells have a homozygous partial deletion of the p53 protein, and lack expression of p53 protein. The cells are able to synthesize the peptide NMB at 0.1 pmol/mg protein, but not the gastrin releasing peptide (GRP).

Another lung carcinoma cell contemplated for use in certain embodiments of the present invention is designated βG H05 (ATCC Number: CRL-5808). This is a classic small cell lung cancer cell line with an epithelial morphology. This line was derived from cells recovered from pleural effusion taken from a patient after chemotherapy. βG H05 expresses elevated levels of the 4 biochemical markers of SCLC: neuron specific enolase, the brain isoenzyme of creatine kinase, L-dopa carboxylase and bombesin-like immunoreactivity. The cells express the c-kit gene as well as the L-myc gene, and L-myc is amplified. The cells express easily detectable levels of p53 mRNA compared to levels found in normal lung.

Also contemplated for use is βG H06 (ATCC Number: CRL-5815), having an epithelial morphology; these cells produce neuromedin B (NMB). This line was derived from tissue taken prior to therapy. This is the best differentiated of the available bronchial carcinoid lines. The cells express easily detectable levels of p53 mRNA compared to levels found in normal lung. The cells are able to synthesize the peptide NMB (at 0.1 pmol/mg protein), but not the gastrin releasing peptide (GRP). The cell line secretes a parathyroid hormone-like protein which is calcium stimulated through a protein kinase C pathway. Further, growth of βG H06 cells is inhibited by epidermal growth factor (EGF) receptor monoclonal antibodies.

Another classic small cell lung cancer cell line is βG H07 (ATCC Number: CRL-5804). This line was derived from cells recovered from pleural effusion obtained from a patient prior to therapy, and expresses elevated levels of the 4 biochemical markers of SCLC: neuron-specific enolase, the brain isoenzyme of creatine kinase, L-dopa carboxylase and bombesin-like immunoreactivity. Only trace amounts of the retinoblastoma susceptibility gene (RB) mRNA, were detected. RB protein was not detected. The cells express the c-kit gene as well as the N-myc gene. N-myc is not amplified. The cells are not able to synthesize the peptide neuromedin B (NMB) or the gastrin-releasing peptide (GRP). They express easily detectable levels of p53 mRNA compared to levels found in normal lung. These cells are also useful for transfection studies.

βG H08 are carcinoma cells isolated from a stage 3A squamous cell lymph node carcinoma (ATCC Number: CRL-5867). βG H09 are derived from an atypical lung carcinoid and are available form the ATCC (CRL-5838). βG H10 cell line is a commercially available cell line derive from lung carcinoma (ATCC Number CCL-185) Another similar cell line is

ATCC number CCL-185.1 derived from CCL-185 which was initiated through explant culture of lung carcinomatous tissue. CCL-185.1 are adapted to growth in serum-free medium.

βG H11 cells may be obtained form ATCC (number HTB-9) and are derived from a bladder carcinoma. βG H13 (ATCC Number: CRL-2139) are from a primitive neuroectodermal brain tumor. The cells express CCK specific mRNA and synthesize considerable quantities of variably processed CCK prohormone.

ATCC Number: CCL-249 are designated herein as βG H14 and are derived from a colon adenocarcinoma. This is one of 14 colorectal carcinoma cell lines derived from a well differentiated sigmoid tumor from a patient prior to therapy. The line was initially grown in medium with fetal bovine serum, but was later adapted to growth in the chemically defined medium ACL-4. Floating aggregates produce tubuloglandular structures lined by columnar epithelia.

βG H15 are from a colorectal carcinoma (ATCC Number: CCL-253) and have an epithelial phenotype. This line was derived from a metastasis to the abdominal wall obtained from a patient after treatment with 5-fluorouracil. βG H16 are the same as the commercially available cell line of ATCC Number: CRL-5974. These are gastric carcinoma cells that express the surface glycoproteins carcinoembryonic antigen (CEA) and TAG 72 and the muscarinic cholinergic and vasoactive intestinal peptide (VIP) receptors, but lack gastrin receptors

ATCC Number: HTB-10 are the cells referred to herein as βG H18. These cells are derived from a neuroblastoma cell line and is one of two cell lines (see also ATCC HTB-11) of neurogenic origin. βG H19 or ATCC Number: HTB-184 are small cell lung carcinoma cells of an extrapulmonary origin and are from an adrenal metastasis in an adult. The cells produce easily detectable p53 mRNA at levels comparable to those in normal lung tissue.

βG H20 (ATCC Number: HTB-177) is a large cell carcinoma cell line derived from the pleural fluid of a patient with large cell cancer of the lung. The cells stain positively for keratin and vimentin but are negative for neurofilament triplet protein. The line expresses some properties of neuroendocrine cells, is relatively chemosensitive and can be cloned in soft agar (with or without serum).

βG H21 (ATCC Number: CRL-2195) is yet another small cell lung carcinoma (SCLC) cell that is contemplated for use as a starting cell in certain aspects of the present invention. It can grow as suspension and loosely adherent culture, and is a biochemically stable continuously cultured cell line that has retained important features of SCLC. The line was derived from a non-encapsulated primary lung tumor from the apical portion of the upper lobe of the left lung. This cell line is an unusual undifferentiated large cell variant of small cell lung carcinoma. It has the morphology of a variant, but the biochemical properties of a classic SCLC. Electron microscopy revealed the presence of gland formation and intracytoplasmic lamellar bodies. The cells have neuroendocrine markers L-dopa decarboxylase and dense core secretory granules.

βG H23 is a long-term tissue culture cell line derived from a metastatic human carcinoid tumor of the pancreas (Evers *et al.*, 1991; Parekh *et al.*, 1994). This cell line is also known as BON (Evers *et al.*, 1991); tumors derived from this cell line are histologically identical to the original tumor. The cells have significant amounts of neurotensin, pancreastatin, and serotonin (5-HT), as demonstrated in the cells by radioimmunoassay (RIA), and chromogranin A, bombesin, and 5-HT, as confirmed by immunocytochemistry. Further, the cells possess neurosecretory granules and functional receptors for acetylcholine, 5-HT, isoproterenol, and somatostatin. BON cells possess a specific transport system for uptake of 5-HT from the medium; this uptake system may be a route for regulation of autocrine effects of 5-HT on carcinoid cells (Parekh *et al.*, 1994). This unique human carcinoid tumor cell line provides an exemplary starting material for the bioengineering described herein and possess intracellular mechanisms ideally adapted for secretagogue action in the release of amines and peptides.

Yet another starting cell that is contemplated for use in the present invention is designated βG H25 (ATCC Number: HB-8065), which is derived from a hepatoblastoma.

This cell line produces α-fetoprotein, albumin, α-2-macroglobulin, α-1-antitrypsin, transferrin; α-1-antichymotrypsin, haptoglobin, ceruloplasmin, plasminogen, and demonstrates decreased expression of apoA-I mRNA and increased expression of catalase mRNA in response to gramoxone (oxidative stress), complement (C4), C3 activator, fibrinogen, α-1 acid glycoprotein, α-2-HS-glycoprotein, β-lipoprotein and retinol binding protein.

D. Neuroendocrine Cell Types

Table 1 shown above (Pearse and Takor, 1979; Nylen and Becker, 1995), while not a complete list, is exemplary of the types of cells contemplated for use in the present invention. β-cells and pituitary cells are preferred for use in the present invention, with β-cells being more preferred. Additional cell types useful in the present invention will be readily known to those of skill in the art.

In addition to β-cells, pituitary cells are preferred for use with this invention. In general, pituitary cells may allow for higher efficiency of transformation as culture conditions have been reported for promoting the proliferation of rodent pituitary cells *in vitro* (Nicol *et al.*, 1990). The inventors contemplate establishing similar conditions for human pituitary cells which will allow for retroviral infection and provide a means for efficiently introducing transforming genes.

Pituitary cells may have an advantage for use in cell-based therapies of IDDM as there is a suggestion that this cell type can survive and sustain secretory function in autoimmune disease. The POMC promoter was used to drive expression of insulin in the cells of the intermediate lobe of transgenic nonobese diabetic (NOD) mice. Such cells were resistant to autoimmune-dependent destruction even when implanted next to islets in which β-cells were destroyed during the course of the disease (Lipes *et al.*, 1996).

II. Secretory Polypeptides

Expression of one or more proteins or polypeptides that are normally secreted can be engineered into neuroendocrine cells. The cDNA's encoding a number of useful human proteins are available. Examples would include soluble CD-4, Factor VIII, Factor IX, von Willebrand Factor, TPA, urokinase, hirudin, interferons, TNF, interleukins, hematopoietic growth factors, antibodies, albumin, leptin, transferrin and nerve growth factors. Exemplary secretory polypeptides are listed in Table 1 above, and in Tables 3-5 below.

Peptide hormones claimed herein for engineering in neuroendocrine cells are grouped into three classes with specific examples given for each. These classes are defined by the complexity of their post-translational processing. Class I is represented by growth hormone, prolactin and parathyroid hormone. A more extensive list of human peptides that are included in Class I is given in Table 3. These require relatively limited proteolytic processing followed by storage and stimulated release from secretory granules. Class II is represented by insulin and glucagon. A more extensive list of human peptide hormones that are included in Class II are given in Table 4. Further proteolytic processing is required, with both endoproteases and carboxypeptidases processing of larger precursor molecules occurring in the secretory granules. Class III is represented by amylin, glucagon-like peptide I and calcitonin. Again, a more extensive list of Class III human peptide hormones is given in Table 5. In addition to the proteolytic processing found in the Class II peptides, amidation of the C-terminus is required for proper biological function.

TABLE 3

Class I Human Peptide Hormones

Growth Hormone
Prolactin
Placental Lactogen
Luteinizing Hormone
Follicle-stimulating Hormone
Chorionic Gonadotropin
Thyroid-stimulating Hormone
Leptin

TABLE 4

Human Peptide Hormones Processed by Endoproteases from Larger Precursors

Adrenocorticotropin (ACTH)
Angiotensin I and II
β-endorphin
β-Melanocyte Stimulating Hormone (β-MSH)
Cholecystokinin
Endothelin I
Galanin
Gastric Inhibitory Peptide (GIP)
Glucagon
Insulin
Lipotropins
Neurophysins
Somatostatin

TABLE 5

Amidated Human Peptide Hormones

Calcium Metabolism Peptides:

- Calcitonin
- Calcitonin Gene related Peptide (CGRP)
- β-Calcitonin Gene Related Peptide
- Hypercalcemia of Malignancy Factor (1-40) (PTH-rP)
- Parathyroid Hormone-related protein (107-139) (PTH-rP)
- Parathyroid Hormone-related protein (107-111) (PTH-rP)

Gastrointestinal Peptides:

- Cholecystokinin (27-33) (CCK)
- Galanin Message Associated Peptide, Preprogalanin (65-105)
- Gastrin I
- Gastrin Releasing Peptide
- Glucagon-like Peptide (GLP-1)
- Pancreastatin
- Pancreatic Peptide
- Peptide YY
- PHM
- Secretin

TABLE 5 - CONTINUED

- Vasoactive Intestinal Peptide (VIP)

Pituitary Peptides:

- Oxytocin
- Vasopressin (AVP)
- Vasotocin

Enkephalins:

- Enkephalinamide
- Metorphinamide (Adrenorphin)

Others:

Alpha Melanocyte Stimulating Hormone (alpha-MSH)
Atrial Natriuretic Factor (5-28) (ANF)
Amylin
Amyloid P Component (SAP-1)
Corticotropin Releasing Hormone (CRH)
Growth Hormone Releasing Factor (GHRH)
Luteinizing Hormone-Releasing Hormone (LHRH)
Neuropeptide Y
Substance K (Neurokinin A)
Substance P
Thyrotropin Releasing Hormone (TRH)

In still further embodiments of the present invention, the engineered cells may express and/or overexpress one or more enzymes of therapeutic value. Such enzymes include, but are not limited to, adenosine deaminase (*e.g.* Genbank Accession Nos. P55265; U18121; U73107; Z97053; P00813; U75503; DUHUA), galactosidase (*e.g.* Genbank Accession Nos P54803; P51569; P23780; D00039), glucosidase (*e.g.* Genbank Accession Nos P29064 (α-glucosidase), P26208 (β-glucosidase), lecithin:cholesterol acyltransferase (LCAT, *e.g.* Genbank Accession Nos. 729921 (baboon), P04180 (human), XXHUN (human LCAT precursor), X04981), factor IX (*e.g.*, Genbank Accession Nos. P00740 (human) K02402 (human) P00741 (bovine) and A22493), sphingolipase, lysosomal acid lipase (*e.g.*, Genbank

Accession Nos P38571; S41408), lipoprotein lipase (*e.g.*, Genbank Accession No. P06858), hepatic lipase (*e.g.*, Genbank Accession Nos. AF037404; P11150; P07098), pancreatic lipase related protein (*e.g.*, Genbank Accession Nos. P54315; P54317) pancreatic lipase (P16233) and uronidase.

III. Expression Constructs

Recombinant vectors form important further aspects of the present invention. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. Thus, in certain embodiments, expression includes both transcription of a gene and translation of a RNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid, for example, to generate antisense or ribozyme constructs.

Particularly useful vectors are contemplated to be those vectors in which the coding portion of the DNA segment, whether encoding a full length protein or smaller peptide, is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned", "under control" or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The promoter may be in the form of the promoter that is naturally associated with a particular gene, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or polymerase chain reaction (PCR™) technology, in connection with the compositions disclosed herein (PCR technology is disclosed in U.S. Patent 4,683,202 and U.S. Patent 4,682,195, each incorporated herein by reference).

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with a particular gene in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cells.

Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides.

At least one module in a promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

A. Secretory Cell-Specific Promoters

1. Sufficient Specificity

The promoter is required to express the transforming genetic construct to a degree sufficient to effect transformation of a target cell type amongst a population of different cell types such that the transformed target cell results in the generation of a stable human regulated secretory cell.

Promoters can be classified into two groups, ubiquitous and tissue- or cell-specific. Ubiquitous promoters activate transcription in all or most tissues and cell types. Examples of ubiquitous promoters are cellular promoters like the histone promoters, promoters for many metabolic enzyme genes such as hexokinase I and glyceraldehyde-3-phosphate dehydrogenase, and many viral promoters such as the cytomegalovirus promoter (CMVp) and the Rous sarcoma virus promoter (RSVp). In certain aspects of the present invention, these promoters are appropriate for use with the immortalizing constructs described herein, as well as finding use in additional aspects of the present invention. Additional examples of these types of promoters that are contemplated for use in the present invention are listed below in Tables 6 and 7.

Tissue- or cell-specific promoters activate transcription in a restricted set of tissues or cell types or, in some cases, only in a single cell type of a particular tissue. Examples of stringent cell-specific promoters are the insulin gene promoters which are expressed in only a single cell type (pancreatic β-cells) while remaining silent in all other cell types, and the immunoglobulin gene promoters which are expressed only in cell types of the immune system.

Various exemplary promoters are shown above in Table 1 (Pearse and Takor, 1979; Nylen and Becker, 1995). Although not a complete list, these promoters are exemplary of the types of promoters contemplated for use in the present invention. Additional promoters useful in the present invention will be readily known to those of skill in the art.

2. Context Specificity

The promoter may be "context specific" in that it will be expressed only in the desired cell type and not in other cell types that are likely to be present in the population of target cells, *e.g.,* it will be expressed in β-cells, but not in α- or δ-cells, when introduced into intact human islets. In this scenario, an insulin promoter targets the expression of a linked transforming oncogene selectively to β-cells of a human islet preparation even though many other contaminating cell types exist in the preparation.

As the present invention is applicable to the generation of stably transformed neuroendocrine secretory cell lines other than β-cells, other context specific promoters may be employed. For example, the cell-specific prolactin gene promoter can be used to express a linked transforming oncogene selectively to lactotrophs surrounded by all the other cell types present in a pituitary cell preparation.

3. Exemplary Promoters

a. β-Cell-Specific Promoters

It has been documented that the two rat insulin gene promoters, RIP1 (GenBank accession number J00747) and RIP2 (GenBank accession number J00748), as well as the human insulin promoter (HIP; GenBank accession number V00565), direct stringent cell-specific expression of the insulin gene in rodent β-cell insulinoma lines (German *et al.*, 1990), primary islet cells (Melloul *et al.*, 1993), and in β-cells of transgenic mice (Efrat *et al.*, 1988).

As the sequence and position of the functional promoter elements are well conserved between HIP, RIP1 and RIP2, the transcription factors that interact with these elements are likely to be conserved across species. In fact, HIP can direct cell-specific expression of linked genes in rodent β-cell lines and rat primary islets, albeit, at a somewhat lower level than that observed for RIP1 (Melloul *et al.*, 1993). The inventors postulate that RIP1 and RIP2 should function effectively in human β-cells.

Melloul *et al.* (1993) demonstrated that the isolated 50-bp RIP1 FAR/FLAT minienhancer (FF), an essential promoter element for RIP1 activity, could express a linked reporter gene in both adult rat and human islet cells. Furthermore, FF activity could be substantially induced by increased concentrations of glucose in both species of adult islets. Additional results from gel-shift studies strongly suggested that the same or similar β-cell-specific transcription factor(s) from both adult rat and human islet cell nuclear extracts bound to conserved sequences contained within both the RIP1 FF and the analogous region of HIP.

b. Further Neuroendocrine Cell-Specific Promoters

As used herein in the context of the present invention, the term "neuroendocrine cell specific promoter" is used to define a promoter that is specific for one or more types of neuroendocrine cells. Thus, in certain aspects of the present invention, a promoter that specifically expresses of at least a first immortalizing construct in one, two, three, four, a plurality or all neuroendocrine cell types, but not in other, non-neuroendocrine cell types, is contemplated for use.

Exemplary of this class of promoter are the glucagon promoter, GenBank accession number X03991; growth hormone promoter, GenBank accession numbers J03071 and K00470; POMC gene promoter, GenBank accession numbers V01510 and K02406; calcitonin promoter, GenBank accession number X15943; the GIP gene promoter, GenBank accession number M31674, and the α-glycoprotein promoter (Genbank accession number LO5632).

4. Modified Promoters

Promoters can be modified in a number of ways to increase their transcriptional activity. Multiple copies of a given promoter can be linked in tandem, mutations which increase activity may be introduced, single or multiple copies of individual promoter elements may be attached, parts of unrelated promoters may be fused together, or some combination of all of the above can be employed to generate highly active promoters. All such methods are contemplated for use in connection with the present invention.

German *et al.* (1992a) mutated three nucleotides in the transcriptionally important FLAT E box of the rat insulin I gene promoter (RIP), resulting in a three- to four-fold increase in transcriptional activity of the mutated RIP compared to that of a nonmutated RIP as assayed in transiently transfected HIT cells. Also, the introduction of multiple copies of a promoter element from the *E. coli* tetracycline resistance operon promoter were introduced into the CMV promoter, significantly increasing the activity of this already very potent promoter (Liang *et al.*, 1996). Additionally, part of the CMV promoter, which has high but short-lived transcriptional activity in dog myoblasts, was linked to the muscle-specific creatine kinase promoter (MCKp), which has weak but sustained expression in dog myoblasts, resulting in a hybrid promoter that sustained high-level expression for extended periods in dog myoblasts.

5. Multimerized Promoters

Several modified rat insulin promoters (modRIP) containing multimerized enhancer elements have been engineered. The currently preferred modRIP contains six multimerized repeats of a 50 base pair region of the *cis* acting enhancer of RIP, placed upstream of an intact copy of RIP.

These novel promoters have been shown to direct expression of transgenes in stably engineered β-cell lines at levels above those attained with unmodified insulin promoters and, in some cases, approaching the levels achieved with the cytomegalovirus promoter (CMVp). CMVp is one of the strongest activating promoters known, but in a very non-tissue specific manner. Therefore, the present modified rat insulin promoters can be used to direct the tissue specific expression of transforming genes at levels presently achievable only with the non-specific CMVp.

B. Additional Promoters and Other Expression Elements

The particular promoter that is employed to control the expression of a nucleic acid, except in certain embodiments the promoter used to express the one or more immortalizing constructs (as discussed in detail herein), is not believed to be critical, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, as human cells are being targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter. Preferred promoters include those derived from HSV, including the HNF1α promoter. Another preferred embodiment are the tetracycline controlled promoters.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. Tables 6 and 7 below list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of a particular gene, except as discussed above when a promoter that is specific for expression in a particular cell type is required or desired. This list is not intended to be exhaustive of all the possible elements involved in the promotion of transgene expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a transgene. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

Table 6 - Promoter and Enhancer Elements

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji *et al.,* 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto and Baltimore, 1989; Redondo *et al.*; 1990
HLA DQ a and DQ β	Sullivan and Peterlin, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1988
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-DRa	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Ornitz *et al.*, 1987
Metallothionein	Karin *et al.*, 1987; Culotta and Hamer, 1989

TABLE 6 - CONTINUED

Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987
Albumin Gene	Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β-Globin	Trudel and Constantini, 1987
e-fos	Cohen *et al.*, 1987
c-HA-ras	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh *et al.*, 1990
$\alpha_{\text{1-Antitrypain}}$	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988

TABLE 6 - CONTINUED

Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens and Hentschel, 1987; Glue *et al.*, 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989
Cytomegalovirus	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

Table 7 - Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b
SV40	Phorbol Ester (TPA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	Ela, SV40 Large T Antigen	Taylor *et al.*, 1989; Taylor and Kingston, 1990a, b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel *et al.*, 1989

TABLE 7 - CONTINUED

Thyroid Stimulating Hormone a Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

Turning to expression, once a suitable clone or clones have been obtained, whether they be cDNA based or genomic, one may proceed to prepare an expression system. The engineering of DNA segment(s) for expression in human neuroendocrine cells may be performed by techniques generally known to those of skill in recombinant expression. It is believed that a number of different expression systems may be employed in the expression of proteins and peptides in the present invention.

Both cDNA and genomic sequences are suitable for expression in human neuroendocrine cells, as these cells will generally process the genomic transcripts to yield functional mRNA for translation into protein. Generally speaking, it may be more convenient to employ as the recombinant gene a cDNA version of the gene. It is believed that the use of a cDNA version will provide advantages in that the size of the gene will generally be much smaller and more readily employed to transfect the targeted cell than will the genomic version of the gene, which will typically be up to an order of magnitude larger than the cDNA version of the gene. However, the inventors do not exclude the possibility of employing a genomic version of a particular gene where desired.

In expression, one will also typically desire to incorporate into the transcriptional unit an appropriate polyadenylation site (*e.g.*, 5'-AATAAA-3') if one was not contained within the original cloned segment. Typically, the poly A addition site is placed about 30 to 2000 nucleotides "downstream" of the termination site of the protein at a position prior to transcription termination. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal and the bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon and adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

It is proposed that a particular protein may be co-expressed with one or more additional selected protein(s) or polypeptide(s), wherein the proteins may be co-expressed in the same cell or one or more particular gene(s) may be provided to a human neuroendocrine cell that already has another selected protein. Co-expression may be achieved by co-transfecting the cell with two or more distinct recombinant vectors, each bearing a copy of the respective DNA. Alternatively, a single recombinant vector may be constructed to include the coding regions for the two or more proteins, which could then be expressed in cells transfected with the single vector. In either event, the term "co-expression" herein refers to the expression of two or more selected proteins in the same recombinant cell.

In certain embodiments of the invention, the use of internal ribosome binding site (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′ methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988; Jang *et al.*, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

As used herein, the terms "engineered" and "recombinant" cells are intended to refer to a human neuroendocrine cell into which an exogenous DNA segment or gene, such as a cDNA or genomic version of a gene has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced exogenous DNA segment or gene. Engineered cells are thus cells having a gene or genes introduced through the hand of man. Recombinant cells include those having an introduced cDNA or genomic version of a gene, and also include genes positioned adjacent to a promoter not naturally associated with the particular introduced gene.

To express a selected protein, whether mutant or wild-type, in accordance with the present invention one would prepare an expression vector that comprises a wild-type or mutant protein-encoding nucleic acid under the control of one or more promoters. To bring a coding sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded recombinant protein. This is the meaning of "recombinant expression" in this context.

Many standard techniques are available to construct expression vectors containing the appropriate nucleic acids and transcriptional/translational control sequences in order to achieve protein or peptide expression in human neuroendocrine cells. Suitable expression vectors include, but are not limited to, bacteriophage, plasmid, cosmid, viral, or artificial chromosome (including, but not limited to, bacterial artificial chromosome (BAC), yeast artificial chromosome (YAC) and human artificial chromosome (HAC)) expression vectors.

Expression vectors for use in mammalian such cells ordinarily include an origin of replication (as necessary), a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences. The origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (*e.g.*, Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

The promoters may be derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter). Further, it is also possible, and may be desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the human neuroendocrine cell systems.

A number of viral based expression systems may be utilized, for example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the *Hin*dIII site toward the *Bgl*I site located in the viral origin of replication.

In cases where an adenovirus is used as an expression vector, the coding sequences may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a desired protein in infected hosts.

For long-term, high-yield production of a recombinant protein, stable expression is preferred. For example, cell lines that stably express constructs encoding a desired protein may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with vectors controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, *etc.*), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

In certain aspects of the present invention, specific cells are tagged with specific genetic markers to provide information about the fate of the tagged cells. Therefore, the present invention also provides recombinant candidate screening and selection methods which are based upon whole cell assays and which, preferably, employ a reporter gene that confers on its recombinant hosts a readily detectable phenotype that emerges only under conditions where a general DNA promoter positioned upstream of the reporter gene is functional. Generally, reporter genes encode a polypeptide (marker protein) not otherwise produced by the host cell which is detectable by analysis of the cell culture, *e.g.*, by fluorometric, radioisotopic or spectrophotometric analysis of the cell culture. In other aspects of the present invention, a genetic marker is provided which is detectable by standard genetic analysis techniques, such as DNA amplification by PCR™ or hybridization using fluorometric, radioisotopic or spectrophotometric probes.

Exemplary enzymes for screening include esterases, phosphatases, proteases (tissue plasminogen activator or urokinase) and other enzymes capable of being detected by their activity, as will be known to those skilled in the art. Contemplated for use in the present invention is green fluorescent protein (GFP) as a marker for transgene expression (Chalfie *et al.*, 1994). The use of GFP does not need exogenously added substrates, only irradiation by near UV or blue light, and thus has significant potential for use in monitoring gene expression in living cells.

Other particular examples are the enzyme chloramphenicol acetyltransferase (CAT) which may be employed with a radiolabelled substrate, firefly and bacterial luciferase, and the bacterial enzymes β-galactosidase and β-glucuronidase. Other marker genes within this class are well known to those of skill in the art, and are contemplated for use in the present invention.

Another class of reporter genes which confer detectable characteristics on a host cell are those which encode polypeptides, generally enzymes, which render their transformants resistant against toxins. Examples of this class of reporter genes are: the *neo* gene (Colberre-Garapin *et al.*, 1981) which protects host cells against toxic levels of the antibiotic G418; the gene conferring streptomycin resistance (U. S. Patent 4,430,434); the gene conferring hygromycin B resistance (Santerre *et al.*, 1984; U. S. Patents 4,727,028, 4,960,704 and 4,559,302); a gene encoding dihydrofolate reductase, which confers resistance to methotrexate (Alt *et al.*, 1978); gpt, which confers resistance to mycophenolic acid; the enzyme HPRT; genes that confer resistance to puromycin, hygromycin, zeocin, phleomycin, blasticidin or histidinol; the herpes simplex virus thymidine kinase gene; and many others well known in the art (Kaufman, 1990).

It is contemplated that a protein may be "overexpressed", *i.e.*, expressed in increased levels relative to its natural expression in cells. Such overexpression may be assessed by a variety of methods, including radiolabeling and/or protein purification. However, simple and direct methods are preferred, for example, those involving SDS/PAGE and protein staining or western blotting, followed by quantitative analyses, such as densitometric scanning of the resultant gel or blot. A specific increase in the level of the recombinant protein or peptide in comparison to the level in natural cells is indicative of overexpression, as is a relative abundance of the specific protein in relation to the other proteins produced by the host cell and, *e.g.*, visible on a gel.

IV. Immortalizing Genetic Constructs

A. Oncogenes and Mutant Tumor Suppressors

Exemplary immortalizing genes and constructs are listed herein in Table 8. Any one or more of the genes listed therein may be used in the context of the present invention. Where two or more immortalizing genes are provided to a human neuroendocrine cell, it may be preferable to provide genes from different functional categories, such as those that perturb signal transduction, affect cell cycle, alter nuclear transcription, alter telomere structure or function, inhibit apoptosis, or that exert pleiotropic activities. It will be understood that the genes listed in Table 8 are only exemplary of the types of oncogenes, mutated tumor suppressors and other immortalizing genetic constructs and elements that may be used in this invention. Further immortalizing genes and constructs will be known to those of ordinary skill in the art.

TABLE 8

Exemplary Proliferative/Immortalizing Agents and Mutant Tumor Suppressors

I. PROLIFERATIVE/IMMORTALIZING AGENTS	MODE OF ACTION
tyrosine kinases, both membrane-associated and cytoplasmic forms, such as Src family, Jak/Stats, Ros, Neu, Fms, Ret, abl, Met serine/threonine kinases: Mos, Raf, protein kinase C, PIM-1 growth factor and receptors: platelet derived growth factor (PDGF), insulin-like growth factor (IGF-1), insulin receptor substrate (IRS-1 and IRS-2), Erb family, epidermal growth factor (EGF), growth hormone, hepatocyte growth factor (HGF) basic fibroblast growth factor (bFGF) small GTPases (G) proteins including the ras family, rab family, and Gs_{α} receptor-type tyrosine phosphatase IA-2	perturb signal transduction
cyclin-dependent protein kinases (cdk), classes A - E; members of the cyclin family such as cyclin D	affect cell cycle

TABLE 8 - CONTINUED

cyclin-dependent protein kinases (cdk), classes A - E; members of the cyclin family such as cyclin D	affect cell cycle
Myc family members including c-myc, N-myc, and L-myc; Rel family members including NF-kappaB; c-Myb, Ap-1, fos, jun, insulinoma associated cDNA (IA-1), ErbB-1, PAX gene family	alter nuclear transcription
telomerase	lengthens telomeres of chromosomes
bcl-2 and family members including Bcl-xl, Mcl-1, Bak, A1, A20 inhibitors of interleukin-1b-converting enzyme and family members	inhibit apoptosis
viral proteins such as SV40 and polyoma large T antigens, SV40 temperature sensitive large T antigen, adenovirus E1A and E1B, papilomavirus E6 and E7	pleiotropic activities
II. MUTANT TUMOR SUPPRESSORS	
p53, retinoblastoma gene (Rb), Wilm's tumor (WT1), bax alpha, interleukin-1b-converting enzyme and family, MEN-1 gene (chromosome 11q13), neurofibromatosis, type 1 (NF1), cdk inhibitor p16, colorectal cancer gene (DCC), familial adenomatosis polyposis gene (FAP), multiple tumor suppressor gene (MTS-1), BRCA1, BRCA2	failure to promote apoptosis

1. Perturbation of Signal Transduction

Representative members of this class are genes or cDNAs encoding tyrosine kinases, serine/threonine kinases, growth factors and receptors, small GTPases, and receptor-type tyrosine phosphatase IA-2. Exemplary of the members preferred for use in the present invention is *neu* (also known as *her2* or *erbB-2*; GenBank accession numbers M11730, X03363, U02326 and S57296). *neu* was discovered as an oncogene in breast cancer, but it is also found in other forms of cancer. *neu* appears to be a member of the receptor tyrosine kinase family. Preferred for use in certain aspects of the present invention are insulin receptor substrate-1 (IRS-1; Genbank accession number S62539) and the insulin receptor substrate-2 (IRS-2; Genbank accession number AB000732). Also preferred is hepatocyte growth factor receptor (HGFr, also known as scatter factor receptor; GenBank accession number U11813).

This is an example of a receptor, either endogenously present or expressed from a recombinant adenovirus, that is used to stimulate proliferation of a target cell population. Other preferred members are insulin-like growth factor 1 receptor (GenBank accession number X04434 and M24599), and GTPase Gs_{α} (GenBank accession numbers X56009, X04409). Gs_{α} is associated with pituitary tumors that secrete growth hormone, but not other neuroendocrine or endocrine tumors.

2. Affect Cell Cycle

Exemplary for use in the present invention is cyclin D1 (also known as PRAD or *bcl-1*; GenBank accession numbers M64349 and M73554). This is an oncogene primarily associated with parathyroid tumors.

3. Alteration of Nuclear Transcription

Exemplary is c-*myc* (GenBank accession numbers J00120, K01980, M23541, V00501, X00364.

4. Alteration of Telomere Structure

Telomeres occur at the end of linear chromosomes, and are required for the completion of DNA replication and the maintenance of genetic stability (Shay, 1997; Greider, 1998). Telomere length is variable in cell types and lineages. In general, cells with the capacity for unlimited or indefinite replication (most cancer cells, cancer cell lines, and embryonic tissues) maintain long telomeres, whereas a loss in telomere length is accompanied by cellular senescence as exhibited by most somatic cell types of adult tissues. Telomere length is determined by the activity of telomerase, a ribonucleoprotein enzyme composed of at least two components: TERC, the RNA component; and TERT, the catalytic subunit of the enzyme. Telomerase adds hexameric repeats (TTAGGG) to the telomeric end of chromosomes. The tight association between telomere length and the capacity for cellular division has led to the hypothesis that telomerase may function as the molecular "clock" that determines the proliferative potential of a given cell. Consequently, telomerase has received attention as an agent in aging and as a novel target in the development of cancer therapies. The inventors contemplate the use of the catalytic subunit of telomerase, TERT, to immortalize human pancreatic β-cells.

The importance of TERT in cellular proliferation has been demonstrated by several groups. Counter *et al.* (1998) showed that expression of TERT in telomerase-negative cells resulted in levels of telomerase activity that were comparable to those observed in immortal telomerase-positive cells. Likewise, retroviral-mediated expression of TERT in human fibroblasts resulted in the elongation of telomere length and an extended life span (Vaziri and Benchimol, 1998). In a similar study, TERT was stably expressed in human fibroblasts using plasmid transfections. Telomerase-positive cells sustained the capacity for indefinite cellular division, and in addition, maintained a normal karyotype (Bodnar *et al.*, 1998). These studies of telomerase-positive versus telomerase-negative cell populations have been complemented by studies of human cancers, with increased telomerase activity occurring in about 90% of tumors. Several studies suggest that TERT expression may be the rate-limiting step in many cases of cellular immortalization (Kallakury *et al.*, 1997; Kolquist *et al.*, 1998; Tsutsumi *et al.*, 1997).

Described herein are several novel strategies for overcoming the biological obstacles associated with establishing stable human neuroendocrine cells, for example human β-cell lines. One presently preferred strategy is a two-step immortalization strategy, in which a first transgene induces or increases the rate of cellular proliferation, and the additive effects of a second transgene provides stably immortalized cell lines. Certain preferred embodiments utilize the expression of human TERT (GenBank accession numbers: AF018176 and AF015950) either as the first or second immortalizing product or component, or the first and second immortalizing product or component. In certain aspects of the invention, the expression of TERT alone is sufficient to provide human insulinoma cells with unlimited capacity for proliferation.

As described herein, in certain aspects of the invention the directed expression of TERT is afforded through the use of tissue-specific and cell-specific promoters. Promoters specific for the pancreatic β-cell include the insulin promoter and modified derivatives of this promoter that maintain specificity yet have stronger transcriptional activity. In certain aspects of the invention utilizing TERT in the immortalization of human β-cells, TERT is amplified by RT-PCR from the mRNA of a human cell line known to have telomerase activity. Following cloning into plasmid vectors and DNA sequencing to confirm the fidelity of replication, TERT is subcloned into plasmids for β-cell specific expression. Expression of TERT can be achieved by any of the methods described herein, including virally-mediated expression or expression achieved through plasmid transfections.

As discussed in detail herein, in certain aspects of the invention full telomerase catalytic activity will not need to be present in order to generate an immortalized human neuroendocrine cell. Thus, constructs with less than full telomerase catalytic activity will have utility in certain embodiments of the present invention. One of ordinary skill in the art will be readily able to test the catalytic activity of telomerase using any of a number of assays that have been described in the art. For example, U.S. Patent Nos. 5,629,154 and 5,648,215 (incorporated herein by reference in their entirety) describe a telomeric repeat amplification protocol (TRAP) assay that may be used to determine the telomerase activity in a particular sample. A commercially available kit is available to determine telomerase activity (TRAP-eze Telomerase Detection Kit; Oncor, Inc., Gaithersburg, MD).

In a general sense, the method comprises preparing a cell extract from a cell sample, placing an aliquot of the cell extract in a reaction mixture comprising a telomerase substrate lacking a telomeric repeat sequence and a buffer in which telomerase can catalyze extension of the telomerase substrate by addition of telomeric repeat sequences, adding to the reaction mixture a primer comprising a sequence sufficiently complementary to a telomeric repeat to hybridize specifically to the telomeric repeat under conditions such that if an extended telomerase substrate is present in the reaction mixture, the primer will hybridize to the extended telomerase substrate and extend to form a complementary copy of the extended telomerase substrate. The presence of telomerase activity is correlated with the presence of duplex DNA molecules comprising an extended telomerase substrate bound to an extended primer, and the absence of telomerase activity is correlated with the absence of such duplex DNA molecules.

The DNA polymerase is preferably a thermostable DNA polymerase. Using such a polymerase, multiple cycles of primer extension can be carried out. Other suitable methods to form the complementary copy of the extended telomerase substrate include the ligase chain reaction (Barany, 1991), nucleic acid sequence-based amplification (Compton, 1991), self-sustained sequence replication (Guatelli *et al.*, 1990), strand displacement amplification (Walker *et al.*, 1992), and branched DNA signal amplification (Urdea, 1994).

Alternatively, human telomerase activity may be determined by measuring the rate of elongation of an appropriate repetitive sequence (primer), having 2 or more, usually 3 or more, repeats of the telomere unit sequence, TTAGGG. The sequence is labeled with a specific binding pair member at a convenient site, *e.g.*, internally or at the 5'-terminus, which specific binding pair member allows for separation of extended sequences. By using one or more radioactive nucleotide triphosphates or other labeled nucleotide triphosphate the incorporated radioactivity can be measured as cpm per unit weight of DNA as a function of unit of time, thus providing a quantitative measure of telomerase activity. Any other detectable signal and label may also be used, for example fluorescent or enzymatic labels, as described in detail herein.

The activity may be measured using cytoplasmic extracts, nuclear extracts, lysed cells, whole cells, and the like. The particular sample which is employed and the manner of pretreatment will be primarily one of convenience. The pretreatment will be carried out under conditions which avoids denaturation of the telomerase, so as to maintain the telomerase activity. The primer sequence will be selected or labeled so as to allow ready separation of the elongated sequence, which represents the telomerase activity of the sample, from any other DNA present in the sample. The nucleotide triphosphates which may be employed may include at least one nucleotide triphosphate which is labeled. The label will usually be radiolabel, but other labels may also be present. The labels may include specific binding pair members, where the reciprocal member may be labeled with fluorescers, enzymes, or other detectable label, as described in detail herein. Alternatively, the nucleotide triphosphates may be directly labeled with other labels, such as fluorescent labels.

The sequence elongation usually will be carried out at a convenient temperature, generally from about 20°C to 60°C, and for a time sufficient to allow for at least about 100 bp to be added on the average to the initial sequence, generally about 30-90 minutes. After the incubation time to allow for the telomerase catalyzed elongation, the reaction may be terminated by any convenient means, such as denaturation, *e.g.*, heating, addition of an inhibitor, rapid removal of the sequence by means of the label, and washing, or the like. The separated DNA may then be washed to remove any non-specific binding DNA, followed by a measurement of the label by any conventional means.

Other techniques for measuring telomerase activity can use antibodies specific for the telomerase protein, where one may determine the amount of telomerase protein in a variety of ways. For example, one may use polyclonal antisera bound to a surface or a monoclonal antibody for a first epitope bound to a surface, and labeled polyclonal antisera or labeled monoclonal antibody to a second epitope dispersed in a medium, where one can detect the amount of label bound to the surface as a result of the telomerase protein or subunit thereof bridging between the two antibodies. Alternatively, one may provide for primers to the telomerase RNA and using reverse transcriptase and the polymerase chain reaction, determine the presence and amount of the telomerase RNA as indicative of the amount of telomerase present in the cells. Also, *in situ* hybridization can be used to determine the expression of the catalytic subunit of telomerase (TERT) at the single-cell level (Kolquist *et al.*, 1998). The determination of telomerase activity by any of the above techniques may be used to determine whether a cell is immortalized.

5. Inhibitors of Apoptosis

Preferred for use is *bcl-2* (distinct from *bcl-1*, cyclin D1; GenBank accession numbers M14745, X06487). Overexpression of this oncogene was first discovered in T cell lymphomas. *bcl-2* functions as an oncogene by binding and inactivating Bax, a protein in the apoptotic pathway.

6. Pleiotropic Activities

Preferred from this class is SV40 large T antigen (TAG; GenBank accession number J02400), SEQ ID NO:1 (nucleic acid) and SEQ ID NO:2 (peptide sequence). Also preferred are temperature sensitive large T antigen, SEQ ID NO:3 (nucleic acid) and SEQ ID NO:4 (peptide sequence) and human papilloma virus E6 (hpv E6; Genbank accession numbers X67160, A06328, V01116, X03321) and E7 (hpv E7; Genbank accession numbers A06328, V01116, X03321).

7. Failure to Promote Apoptosis

Preferred are mutant p53, retinoblastoma and MEN-1 (GenBank accession number U93236) genes. Most forms of cancer have reports of p53 mutations. Inactivation of p53 results in a failure to promote apoptosis. With this failure, cancer cells progress in tumorogenesis rather than in programmed cell death. A short list of cancers and mutations found in p53 is: ovarian (GenBank accession numbers S53545, S62213, S62216); liver (GenBank accession numbers S62711, S62713, S62714, S67715, S72716); gastric (GenBank accession numbers S63157); colon (GenBank accession numbers S63610); bladder (GenBank accession numbers S85568, S85570, S85691); lung (GenBank accession numbers S41969, S41977); and glioma (GenBank accession numbers S85807, S85712, S85713).

8. Multiple Immortalizing Units

In further preferred embodiments, the invention contemplates the use of several immortalizing gene constructs in combination. As an example of this embodiment, the immortalizing genetic construct may include more than one operative immortalizing unit, or more than one construct can be supplied.

B. Growth Factor Receptor Genes and Growth Factors

In still further preferred embodiments, the invention contemplates the use of one or more growth factor receptor genes and/or one or more growth factor genes as the immortalizing elements.

As an example of this embodiment, human β-cells are infected with a recombinant adenovirus that provides overexpression of growth hormone receptor (GenBank Accession Nos. J04811 and X06562) controlled by the modRIP (or modHIP) promoter. β-cells cultured in a defined medium would then be stimulated to proliferate by the addition of growth hormone to the medium. The replicating population of β-cells are then transformed by retroviral constructs that will result in stable expression of growth hormone receptor or an alternate immortalizing gene. The use of other growth promoting genes such as IGF-1 receptor (and its ligand in the medium), chicken growth hormone (cGH) and chicken growth hormone receptor (cGHr), where the cGH is specific for the cGHr, and/or the signaling substrate of growth factor receptors (such as IRS-2 in the case of IGF-1 receptor) could similarly be used to achieve growth and immortalization.

V. DNA Delivery

In certain embodiments of the invention, the nucleic acid encoding the one or more immortalizing product(s), the one or more secreted protein or polypeptide, or any additional protein or polypeptide may be stably integrated into the genome of the cell. In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the immortalization and/or additional expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of immortalization and/or expression construct employed. All delivery methods are contemplated for use in the context of the present invention, although certain methods are preferred, as outlined below.

A. Transfection

In order to effect expression of one or more immortalizing or other expression construct, the construct must be delivered into a cell. As described below, the preferred mechanism for delivery is *via* viral infection, where the construct is encapsidated in an infectious viral particle. However, several non-viral methods for the transfer of one or more immortalizing or other expression constructs into cultured mammalian cells also are contemplated by the present invention. In one embodiment of the present invention, the expression construct may consist only of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned which physically or chemically permeabilize the cell membrane.

1. Liposome-Mediated Transfection

In a further embodiment of the invention, the one or more immortalizing or other expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated is an expression construct complexed with Lipofectamine (Gibco BRL).

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987). Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1.

Melloul *et al.* (1993) demonstrated transfection of both rat and human islet cells using liposomes made from the cationic lipid DOTAP, and Gainer *et al.* (1996) transfected mouse islets using Lipofectamine-DNA complexes.

2. Electroporation

In certain embodiments of the present invention, the one or more immortalizing or other expression construct is introduced into the cell *via* electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge.

Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter *et al.*, 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa *et al.*, 1986) in this manner.

Examples of electroporation of islets include Soldevila *et al.* (1991) and PCT application WO 91/09939.

3. Calcium Phosphate Precipitation or DEAE-Dextran Treatment

In other embodiments of the present invention, the one or more immortalizing or other expression construct is introduced to the cells using calcium phosphate precipitation. Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe *et al.*, 1990).

In another embodiment, the one or more immortalizing or other expression construct is delivered into the cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal, 1985).

4. Particle Bombardment

Another embodiment of the invention for transferring one or more naked DNA immortalizing or other expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Gainer *et al.* (1996) have transfected mouse islets with a luciferase gene/human immediate early promoter reporter construct, using biolistic particles accelerated by helium pressure.

5. Direct Microinjection or Sonication Loading

Further embodiments of the present invention include the introduction of the one or more immortalizing or other expression construct by direct microinjection or sonication loading. Direct microinjection has been used to introduce nucleic acid constructs into *Xenopus* oocytes (Harland and Weintraub, 1985), and LTK$^-$ fibroblasts have been transfected with the thymidine kinase gene by sonication loading (Fechheimer *et al.*, 1987).

6. Adenoviral Assisted Transfection

In certain embodiments of the present invention, the one or more immortalizing or other expression construct is introduced into the cell using adenovirus assisted transfection. Increased transfection efficiencies have been reported in cell systems using adenovirus coupled systems (Kelleher and Vos, 1994; Cotten *et al.*, 1992; Curiel, 1994), and the inventors contemplate using the same technique to increase transfection efficiencies into human islets.

7. Receptor Mediated Transfection

Still further constructs that may be employed to deliver the one or more immortalizing or other expression construct to the target cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis that will be occurring in the target cells. In view of the cell type-specific distribution of various receptors, this delivery method adds another degree of specificity to the present invention. Specific delivery in the context of another mammalian cell type is described by Wu and Wu (1993; incorporated herein by reference).

Certain receptor-mediated gene targeting vehicles comprise a cell receptor-specific ligand and a DNA-binding agent. Others comprise a cell receptor-specific ligand to which the DNA construct to be delivered has been operatively attached. Several ligands have been used for receptor-mediated gene transfer (Wu and Wu, 1987, 1988; Wagner *et al.*, 1990; Ferkol *et al.*, 1993; Perales *et al.*, 1994; Myers, EPO 0273085), which establishes the operability of the technique. In the context of the present invention, the ligand will be chosen to correspond to a receptor specifically expressed on the neuroendocrine target cell population.

In other embodiments, the DNA delivery vehicle component of a cell-specific gene targeting vehicle may comprise a specific binding ligand in combination with a liposome. The nucleic acids to be delivered are housed within the liposome and the specific binding ligand is functionally incorporated into the liposome membrane. The liposome will thus specifically bind to the receptors of the target cell and deliver the contents to the cell. Such systems have been shown to be functional using systems in which, for example, epidermal growth factor (EGF) is used in the receptor-mediated delivery of a nucleic acid to cells that exhibit upregulation of the EGF receptor.

In still further embodiments, the DNA delivery vehicle component of the targeted delivery vehicles may be a liposome itself, which will preferably comprise one or more lipids or glycoproteins that direct cell-specific binding. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. It is contemplated that the one or more immortalizing or other expression constructs of the present invention can be specifically delivered into the target cells in a similar manner.

B. Viral Infection

1. Adenoviral Infection

One of the preferred methods for delivery of the one or more immortalizing or other expression constructs involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a tissue-specific transforming construct that has been cloned therein.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process.

Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3, or both the E1 and E3 regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 kb of extra DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone.

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Recently, Racher *et al.* (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of media. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of media, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The media is then replaced with 50 ml of fresh media and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the media is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector preferred for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the transforming construct at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.* (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus growth and manipulation is known to those of skill in the art, and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

Recombinant adenovirus and adeno-associated virus (see below) can both infect and transduce non-dividing human primary cells. Gene transfer efficiencies of approximately 70% for isolated rat islets have been demonstrated by the inventors (Becker *et al.*, 1994a; Becker *et al.*, 1994b; Becker *et al.*, 1996) as well as by other investigators (Gainer *et al.*, 1996).

2. AAV Infection

Adeno-associated virus (AAV) is an attractive vector system for use in the human cell transformation of the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells in tissue culture (Muzyczka, 1992). AAV has a broad host range for infectivity (Tratschin, *et al.*, 1984; Laughlin, *et al.*, 1986; Lebkowski, *et al.*, 1988; McLaughlin, *et al.*, 1988), which means it is applicable for use with human neuroendocrine cells, however, the tissue-specific promoter aspect of the present invention will ensure specific expression of the transforming construct in aspects of the invention where this is desired or required. Details concerning the generation and use of rAAV vectors are described in U.S. Patent No. 5,139,941 and U.S. Patent No. 4,797,368, each incorporated herein by reference.

Studies demonstrating the use of AAV in gene delivery include LaFace *et al.* (1988); Zhou *et al.* (1993); Flotte *et al.* (1993); and Walsh *et al.* (1994). Recombinant AAV vectors have been used successfully for *in vitro* and *in vivo* transduction of marker genes (Kaplitt, *et al.*, 1994; Lebkowski, *et al.*, 1988; Samulski, *et al.*, 1989; Shelling and Smith, 1994; Yoder, *et al.*, 1994; Zhou, *et al.*, 1994; Hermonat and Muzyczka, 1984; Tratschin, *et al.*, 1985; McLaughlin, *et al.*, 1988) and genes involved in human diseases (Flotte, *et al.*, 1992; Luo, *et al.*, 1994; Ohi, *et al.*, 1990; Walsh, *et al.*, 1994; Wei, *et al.*, 1994). Recently, an AAV vector has been approved for phase I human trials for the treatment of cystic fibrosis.

AAV is a dependent parvovirus in that it requires coinfection with another virus (either adenovirus or a member of the herpes virus family) to undergo a productive infection in cultured cells (Muzyczka, 1992). In the absence of coinfection with helper virus, the wild type AAV genome integrates through its ends into human chromosome 19 where it resides in a latent state as a provirus (Kotin *et al.*, 1990; Samulski *et al.*, 1991). rAAV, however, is not restricted to chromosome 19 for integration unless the AAV Rep protein is also expressed (Shelling and Smith, 1994). When a cell carrying an AAV provirus is superinfected with a helper virus, the AAV genome is "rescued" from the chromosome or from a recombinant plasmid, and a normal productive infection is established (Samulski, *et al.*, 1989; McLaughlin, *et al.*, 1988; Kotin, *et al.*, 1990; Muzyczka, 1992).

Typically, recombinant AAV (rAAV) virus is made by cotransfecting a plasmid containing the gene of interest flanked by the two AAV terminal repeats (McLaughlin *et al.*, 1988; Samulski *et al.*, 1989; each incorporated herein by reference) and an expression plasmid containing the wild type AAV coding sequences without the terminal repeats, for example pIM45 (McCarty *et al.*, 1991; incorporated herein by reference). The cells are also infected or transfected with adenovirus or plasmids carrying the adenovirus genes required for AAV helper function. rAAV virus stocks made in such fashion are contaminated with adenovirus which must be physically separated from the rAAV particles (for example, by cesium chloride density centrifugation). Alternatively, adenovirus vectors containing the AAV coding regions or cell lines containing the AAV coding regions and some or all of the adenovirus helper genes could be used (Yang *et al.*, 1994a; Clark *et al.*, 1995). Cell lines carrying the rAAV DNA as an integrated provirus can also be used (Flotte *et al.*, 1995).

The present invention contemplates infection of the target cells with a recombinant adeno-associated virus (AAV) containing at least a first immortalizing construct driven by a promoter that expresses the immortalizing construct in the human neuroendocrine cell, for example a tissue specific promoter. Recombinant AAV plasmids with RIP driving T antigen have been constructed, and are preferred for use in certain aspects of the present invention.

3. Retroviral Infection

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975). Additional retroviral vectors contemplated for use in the present invention have been described (Osborne *et al.*, 1990; Flowers *et al.*, 1990; Stockschlaeder *et al.*, 1991; Kiem *et al.*, 1994; Bauer *et al.*, 1995, Miller and Rosman, 1989; Miller, 1992; Miller *et al.*, 1993; each incorporated herein by reference).

Concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990). A preferred cell line is the PA317 cell line (Osborne *et al.*, 1990).

A major determinant of virus titer is the number of packagable RNA transcripts per producer cell, which is dependent on the integrated proviral DNA copy number. Packaging cell lines are coated with viral envelope glycoproteins and are thus resistant to infection by virus of the same host range, but not virus of a different host range. This process is called interference. Therefore, recombinant retroviruses can shuttle back and forth between amphotropic and ecotropic packaging cell lines in a mixed culture (referred to as ping-ponging), thus leading to an increase in proviral DNA copy number and virus titer (Bestwick *et al.*, 1988). Some drawbacks to the ping-pong process are that transfer of packaging functions between ecotropic and anphotropic lines can lead eventually to generation of replication-competent helper virus. Also, increasing numbers of cells express both ecotropic and amphotropic envelope proteins and are therefore resistant to further infection. Moreover, cells with large numbers of proviruses are unhealthy. Thus, there is an optimum period during the ping-pong process when virus titer is high and helper virus is absent. This time period is empirically determined and is relatively constant for a given ecotropic plus amphotropic packaging line combination.

4. Other Viral Vectors

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990). Lentivirus vectors are also contemplated for use in the present invention (Gallichan *et al.*, 1998; Miyoshi *et al.*, 1998; Kafri *et al.*, 1999)

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. Chang *et al.* recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

In still further embodiments of the present invention, the nucleic acids to be delivered are housed within an infective virus that has been engineered to express a specific binding ligand. The virus particle will thus bind specifically to the cognate receptors of the target cell and deliver the contents to the cell. A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification can permit the specific infection of hepatocytes *via* sialoglycoprotein receptors.

Another approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled *via* the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

C. Multiple Viral Infection

A further alternative for practicing the present invention is to use adenovirus or AAV infection of primary cells leading to *in vitro* expansion of a primary cell population that is then amenable to stable oncogene transfer by methods requiring cellular proliferation such as retroviral transduction, plasmid transfection of expanding cells (Lipofectin or electroporation), or a second round of adenovirus and/or AAV infection. Another embodiment of the invention is to use alternating AAV and adenovirus infections. Propagation of AAV is dependent upon adenovirus, and using both viruses may lead to more productive infections. Such a method may increase the number of final cells that have oncogenes integrated and expressed.

Multiple, sequential viral infections may allow one of skill in the art to exploit the benefits of various viral delivery systems and avoid their limitations. For example, a limitation of adenoviral gene delivery is that this system affords a very low rate of integration of viral and recombinant DNAs into the host cell genome. Consequently, adenoviral gene expression is diluted when the cells divide and typically is used only for transient gene expression. An advantage that adenoviral gene delivery has over many other viral vectors is that entry of the virus into the cell and the expression of transgenic proteins is not dependent on cellular replication. This benefit of adenoviral gene delivery is in contrast to retroviruses where the integration and sustained expression of virally introduced DNA is dependent on cellular replication.

The coupling of these two viral systems for the transformation of primary tissues minimizes the limitations of each and maximally exploits their distinct biological properties. For example, primary human pancreatic β-cells typically do not divide in culture and are thereby resistant to transformation by immortalizing gene constructs delivered by retroviruses. However, human β-cells can be infected with adenovirus for the purposes of transgenic protein expression.

In a preferred embodiment, human β-cells or pancreatic islets are first be infected with a recombinant adenovirus that provides for the expression of a growth-promoting protein to stimulate cellular division. Cellular replication is monitored by measuring thymidine incorporation or other techniques that have been developed to monitor DNA replication. In addition or alternatively, dividing cells are enriched by FACS. Following the stimulation of cellular replication (about 12-96 hours following adenoviral infection), cells are infected with a recombinant retrovirus that has been engineered to express one or more immortalizing gene products. The genomic DNA of a dividing cell population is susceptible to stable integration by retrovirus and expression of recombinant proteins. This system of sequential and varied viral infections can further be optimized by the use of tissue-specific promoters for transgene expression in designated cell types and the expression of antibiotic resistance markers to selectively enrich for virally infected cells.

VI. Secretory Cell Culture

A. Culture Conditions

Primary cells are expanded by established culture conditions. For example, β-cells can be cultured and even induced to divide as described (Clark and Chick, 1990; Beattie *et al.* 1991; Hayek *et al.* 1995; each incorporated herein by reference).

Human islets isolated by an automated method (Ricordi *et al.*, 1988) are maintained in culture and transformed by the inventive engineered expression of proteins that promote accelerated cell replication. The transformation methods of the invention preferably involve the use of specific culture conditions designed to preferentially promote neuroendocrine cell growth, which are used in conjunction with the stable activation of cell specific gene promoter controlled protein expression.

The culture conditions are achieved by manipulating the following cell culture variables: media growth/survival factors (such as IGF-1, growth hormone, prolactin, PDGF, hepatocyte growth factor, and transferrin), media differentiation factors (such as TGF-β), media lipids, media metabolites (such as glucose, pyruvate, galactose, and amino acids), media serum (percentage serum, serum fraction, species of serum), gaseous exchange (ratio atmospheric O_2:CO_2, and media volume), physical form of the islets prior to plating (whole, dispersed, or cell sorted islet cells), and extracellular substrate for cellular attachment (such as laminin, collagen, matrigel, and HTB-9 bladder carcinoma derived matrix).

Fibroblast growth/survival in culture is eliminated by culturing the islets in defined media using factors (such as IGF-1, cysteine, and growth hormone) to selectively confer a growth/survival advantage to β-cells in preference to fibroblasts. Establishment of fibroblast free cultures will allow prolonged maintenance of human islet β-cells in culture for subsequent infection with adenovirus expression vectors in cases where β-cells are in a non-proliferative state, or retrovirus expression vectors for β-cells which have been induced to proliferate by oncogene expression. Fibroblasts may even be killed by fibroblast-directed toxins.

B. Defined Media

Media comprising one or more growth factors that stimulate the growth of the target neuroendocrine cell and do not substantially stimulate growth of distinct cells in the cell population; *i.e.,* act to induce preferential growth of the target cells rather than faster-growing, more hardy cells in the population, may be used to deplete fibroblasts. Examples include defined serum free conditions used for β-cells (Clark *et al.*, 1990; incorporated herein by reference), or inclusion of growth or differentiation factors known to allow preferential growth of β-cells (WO 95/29989; incorporated herein by reference).

In one particular embodiment, the inventors have developed a media composition that will be particularly useful in the growth and propagation of the cells of the present invention. The rational behind the development of "BetaGene" media had its beginnings with the observation that in bioreactor high density cultures of β-cell line RIN-38, ethanolamine was a rapidly consumed component of the growth medium. An equimolar mixture of ethanolamine-phosphoethanolamine was found to protect RIN-38 β-cells from linoleic acid toxicity (approximately 30 μg/ml in serum-free medium).

Subsequently, it was found that bioreactor cultures of this cell line could be maintained for weeks in the absence of serum when the media was supplemented with a mixture of ethanolamine-phosphoethanolamine, bovine serum albumin, and transferrin. These indications for critical lipid components ensured that other lipid components as lipoic acid, inositol (both indicated in the literature as protective *in vivo*), cholesterol, TWEEN 80 and putrescine would be included in the subsequent media formulations.

A second finding was critical to the determination of the optimal formulation for β-cells. This was the development of a rapid screening method for evaluating the best commercial media formulation. The method entails encapsulating βG18/3E1 cells, a rodent β-cell line engineered to secrete human insulin, in 1.5% alginate beads. β-cells encapsulated in beads are very amenable to serum-free culture, and beads were cultured in different media ± FBS for 3-6 days and insulin secretion was monitored to estimate growth and function.

The serum-free cultures then were returned to the same base media supplemented with FBS, with continued insulin monitoring. The media screened were those most commonly used for culture of primary islets in the literature. Performance of the different media were indicated by the rate and magnitude of functional loss, as well as the rate of recovery and completeness of recovery after return to FBS supplementation. One media, CMRL1066, was clearly inferior, while M199 and a-MEM were fairly equivalent. Media such as F12 and RPMI were not readily evaluated by this approach, due to the low calcium concentration of these media and resultant deterioration of the Ca-alginate hydrogel. The latter were then evaluated as equal mixtures with M199 and MEM. An M199-F12 mixture was determined to be the best performing formulation tested, while an MEM-F12 mixture could be used with at least short term equivalency. Many components of the BetaGene Media are at concentrations that would be found in mixed formulations, while others reflect optimization by the inventors.

Cells in alginate beads have been used routinely to screen media components. This approach has simplified and greatly accelerated screening studies. The use of cells in beads has been refined to include acutely stimulated insulin secretion. This has led to the identification of culture supplements that are critical to maintaining secretory function in β-cells and β-cell lines in the absence of serum (additional lipids minor, BSA and transferrin major).

The knowledge that normal β-cells have high ascorbate concentrations, and that PAM, the enzyme responsible for amidation of such islet peptides as pancreatic polypeptide and amylin, requires ascorbate and Cu^{2+}, led to the inclusion of these components. However, ascorbate is quite unstable in media at 37°C, therefore, a stabilized form of ascorbate (ascorbate-2-phosphate) was screened for dose-dependent deleterious effects on growth and insulin secretion. None was encountered over a wide range of concentrations from 10^{-6} to 10^{-3} M. An intermediate concentration of stabilized ascorbate-2-phosphate was tested for its effect on amidation, using cells engineered to express GLP-1 or amylin. The intermediate concentration (50-100 μM) was found to greatly improve amidation, both in flask and high density scale-up cultures, and was thus identified as the preferred concentration for BetaGene Media.

Bicarbonate was increased in the formulation to provide better pH control for scale-up cultures (such as the CellCube™). Zinc was supplemented because primary β-cells have high concentrations of zinc and several islet enzymes bind Zn^{2+}, and insulin crystal is coordinate with Zn. Finally, glucose concentrations are known to be critical for β-cell culture. One objective of the media development was to derive a formulation that would optimally support primary pancreatic islets as well as β-cell lines. As a result, human islets were used to determine a glucose concentration that could support survival and function of human islets in culture. Glucose in the range of 7 mM (6-8 mM) provided long term survival (months) of human islets, with maintenance of glucose sensing, as indicated by dose-response studies of glucose-induced insulin secretion, and by maintained (and in islets of two donors restored) insulin processing.

Table 9 below provides an exemplary composition of BetaGene media. Further compositions of BetaGene media contemplated for use in the present invention are found in U.S. Patent Application 09/___,___ (entitled "Media for Neuroendocrine Cells", Attorney Docket No. BTGN:056), filed January 11, 1999, the entire disclosure of which is incorporated herein by reference without disclaimer.

TABLE 9

BetaGene Media

COMPONENT	g/liter
AMINO ACIDS	
Alanine	0.025
Arginine·HCl	0.2107
Asparagine·H_2O	0.05
Aspartate	0.03
Cysteine·HCl·H_2O	0.03512
Cystine·2HCl	0.01564
Glutamic acid·$2H_2O$	0.075
Glutamine	0.1
Glycine	0.028755
Histidine·HCl·H_20	0.04188
Hydroxyproline	0.01
Isoleucine	0.0525
Leucine	0.12
Lysine·HCl	0.07
Methionine	0.015
Phenylalanine	0.032
Proline	0.04
Serine	0.025
Threonine	0.048
Tryptophan	0.01
Tyrosine·2Na·$2H_2O$	0.0519
Valine	0.046
SALTS	
$CaCl_2$	0.11661
$CuSO_4·5H_2O$	0.00000249
$FeSO_4·7H_2O$	0.000834
KCl	0.311825

COMPONENT	**g/liter**
$MgSO_4$	0.09767
NaCl	6.8
$NaHCO_3$	2.2
$NaH_2PO_4 \cdot H_2O$	0.07
Na_2PO_4	0.07102
$ZnSO_4 \cdot 7H_2O$	0.000863
VITAMINS	
Ascorbic acid-2-phosphate, Mg salt	0.0181
DL-αTocopherol $PO_4 \cdot 2Na$	0.000005
D-biotin	0.00001
D-Ca Pantothenate	0.000619
Choline chloride	0.00723
Ergocalciferol	0.0001
Folic acid	0.0013
myo-inositol	0.035
Menadione	0.00001
Niacin	0.000025
Niacinamide	0.000025
PABA	0.00005
Pyridoxal·HCl	0.000025
Pyridoxine·HCl	0.000025
Riboflavin	0.0001
Thiamine·HCl	0.001
Vitamin A acetate	0.00014
Vitamin B12	0.001357
OTHER COMPONENTS:	
Cholesterol	0.0001
Deoxyribose	0.00025
Ethanolamine	0.000156
Glucose	1.4

COMPONENT	g/liter
Glutathione (reduced)	0.000025
Linoleic acid	0.0000421
Lipoic acid	0.0001032
Phenol Red	0.01
Phosphorylethanolamine	0.007055
Putrescine·2HCl	0.000161
Ribose	0.00025
Sodium Acetate	0.025
Sodium Pyruvate	0.1101
TWEEN 80	0.01

C. Proliferation

Cells may be induced to proliferate by initial infection with adenovirus or adeno-associated virus (AAV) comprising a gene that induces cellular proliferation, the gene being under the control of a promoter specific for the regulated secretory cell. The cells may alternatively be induced to proliferate by growth on a stimulatory cell matrix.

D. *In vivo* Passage

A potential concern is that the studies of Hayek and associates (WO 95/29989) have indicated that as human islet cell growth is stimulated, insulin content can fall rapidly. If this same phenomenon occurs as β-cell proliferation is stimulated by methods of the present invention, expression of the insulin promoter driven oncogene or of the endogenous insulin gene may also decline. The use of modified RIP promoters with enhanced activity may overcome this concern.

Alternatively, previous investigators have shown that the fall in insulin content experienced in replicating human islet cells can be partially restored by transplantation of the cells in athymic rodents (Neisor *et al.*, 1979; Beattie *et al.*, 1995). Therefore, to complete the transformation process, it may be necessary to expose the cells to the *in vivo* environment.

Cell transplantation studies in nude rats are straightforward and *in vivo* passage can readily be included as a component of human β-cell line generation. The transformed human cells may be placed *in vivo, e.g.,* under kidney capsule of the nude rat, to allow outgrowth of transformed cells. In addition to promoting maintenance of the tissue specific expression of the oncogene in the primary cells, the lack of an immune response in the nude rat is known to allow long term survival and expression of recombinant adenovirus infected cells (Dai *et al.*, 1995; Yang *et al.*, 1994b).

VII. Resultant Immortalized Human Secretory Cells

The final attributes of the cell lines of the present invention are functionally defined as having maintained a regulated secretory pathway, being stable to *in vitro* culture and, preferably, as being amenable to further engineering.

A. Cell Stability

The resultant human secretory or neuroendocrine cell will be "culturable", *i.e.,* it will be capable of growing *in vitro* and producing the desired endogenous secretory polypeptide with a demonstrated regulated secretory pathway.

An "immortalized" or "stable, transformed" human regulated secretory cell in the context of the present invention will be a cell that exhibits *in vitro* growth for at least twenty population doublings (Freshney, 1994).

B. Differentiated Phenotype

The resultant human regulated secretory cell will also maintain the required differentiated phenotype after transformation, *i.e.,* it will exhibit the phenotypic properties of a demonstrable regulated secretory pathway, secretory storage granules, the capacity for peptide processing, and will produce one or more selected endogenous or exogenous secretory polypeptide(s).

VIII. Immortalized Human β-Cells

The resultant immortalized human β-cells of the present invention will exhibit the capacity to grow *in vitro*, with a minimum of at least about 20 population doublings, or preferably, of about 30, about 40, about 50, about 60, about 70, or about 80 population doublings. Even more preferably, the resultant immortalized human β-cells of the invention will have even further increments of population doublings up to and including a completely transformed state wherein the cells grow in perpetuity.

The immortalized human β-cells of the present invention will also exhibit the capacity to produce biologically active human insulin. Biologically active insulin is dependent on proteolytic cleavage of preproinsulin to proinsulin and, finally, insulin, as well as disulphide bond formation. The insulin produced may be comprised entirely of mature insulin; or entirely of the biological precursor of mature insulin, termed proinsulin; or of all possible mixtures of proinsulin, insulin, and processing intermediates that are produced in the course of conversion of proinsulin to insulin.

While the preferred embodiment of the present invention are immortalized cells that produce primarily or exclusively mature insulin, cells that produce proinsulin will also be useful in various embodiments. Such cells are useful *per se*, particularly as any form of insulin can be obtained *in vitro*, purified and converted to mature insulin.

Cells that produce primarily or exclusively immature insulin are also useful in that the capacity to produce mature insulin can be re-engineered into the cells themselves, in which instances the stable cells can then be used *in vivo*. By way of example only, proteases known as PC2 and PC3 that are responsible for the conversion of proinsulin to insulin can be introduced into the immortalized human β-cells by genetic engineering methods, thereby enhancing the efficiency of conversion of proinsulin to insulin.

The immortalized human β-cells of the present invention will generally exhibit a minimal insulin content of about 5 ng/million cells, but may contain as much as, or even more insulin than, normal isolated human islets, which have approximately 1-10 μg/million cells. It will be understood that the cells of the present invention may contain any amount of insulin within the above-specified ranges, such as about 10 ng insulin/million cells, about 50 ng, about 100 ng, about 200 ng, about 500 ng, about 1000 ng (1 μg), about 2 μg, about 5 μg, about 10 μg, about 20 μg, about 50 μg, about 75 μg, up to and including about 100 μg insulin/million cells. It will be understood that any and all integers within these ranges will define an insulin content that may be present within the immortalized, stable human β-cells of the invention.

In further preferred embodiments, the immortalized human β-cells of the present invention may be defined as cells having an insulin content of between about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, up to and including about 100% of normal human islet content, which is about 1-10 μg/million cells.

The immortalized human β-cells of the present invention will preferably exhibit enhanced insulin secretion when exposed to one or more secretagogues selected from IBMX, carbachol, amino acids, and glucose, or when exposed to a secretory "cocktail" of such compounds. The immortalized human β-cells will more preferably exhibit enhanced insulin secretion when exposed to glucose, and will most preferably exhibit enhanced insulin secretion when exposed to 10 mM glucose.

The increase in insulin secretion in response to a non-glucose secretagogue or cocktail thereof should be at least about 1.1 times or about 1.5 times that observed in cells incubated in the absence of the secretagogue or secretory cocktail. However, in preferred embodiments, the increase in insulin secretion in response to a non-glucose secretagogue or cocktail thereof should be at least about double that observed in cells incubated in the absence of the secretagogue or secretory cocktail. In more preferred embodiments, a higher increase will be observed, up to and including a 3-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 300-fold, 500-fold, 750-fold or even about a 1000-fold enhancement.

The immortalized human β-cells of the present invention will preferably exhibit a glucose-stimulated insulin secretion (GSIS) response. This increase in secretion should be at least about 1.1 times or about 1.5 times that observed in cells incubated in the absence of glucose. More preferred are increases in secretion of at least about double that observed in cells incubated in the absence of glucose, with even more preferred increases being higher, up to and including a 3-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 300-fold, 500-fold, 750-fold or even about a 1000-fold enhancement, including all increments therebetween.

In preferred embodiments, glucose responsive insulin secretion will be observed in the range of 1.0 to 20 mM glucose. GSIS response will more preferably occur with a threshold for response of 3-5 mM glucose, with maximal secretion stimulated by 10-20 mM glucose, as occurs in normal human islets. Cell lines with glucose dose responses occurring over a higher or lower range will also have significant utility, given that any regulated insulin production will be useful.

Even where an immortalized cell is intended for use in human therapy, cells that have a GSIS response not originally in the range observed for normal human islets will still be useful. Such cells will be amenable to genetic engineering methods, as embodied in U.S. Patent 5,427,940, and as further disclosed herein, in order to alter the glucose dose response. These methods are contemplated for use in applications with immortalized human cells to achieve the desired glucose concentration dependence. Furthermore, as stated, immortalized human β-cells that are completely lacking glucose responsiveness are also included within the invention, since the known genetic engineering methods (U.S. Patent 5,427,940) can be used to confer glucose sensing in neuroendocrine cell lines previously lacking a glucose response.

IX. Regulation of the Growth of the Resultant Immortalized Cells

There is an advantage to developing human cell lines that do not ultimately express the immortalizing constructs. The immortalizing genetic construct may, therefore, be functionally and/or physically separated from the cell after immortalization. Advantages include generation of cell lines that do not constitutively express oncogenes which can act as tumor antigens *in vivo*, control of growth of the resulting tumor lines for stable *in vivo* use and possibly the control of the differentiated state of the resultant cell line.

A. Functional Separation

1. Temperature-Sensitive Regulation of Oncogene Expression

The use of temperature sensitive oncogenes allows for turning the growth promoting activity on and off. In general, oncogenes that are active at lower than physiological temperatures (*i. e.* 32 to 34°C) and off at physiological or higher temperatures (37 to 39°C) are preferred. Using this approach, immortalized, stable cell lines can be expanded, and further genetic modifications can be made and characterized *in vitro* at the low, permissive temperatures. When placed *in vivo*, these same cell lines will be exposed to the non-permissive temperature, and will not grow. As an example of an oncogene with these traits, a temperature sensitive version of the SV40 virus was isolated and shown to have a mutation in the coding region of the large T antigen gene (Bourre and Sarasin, 1983).

2. Conditional Expression

Promoters capable of driving expression of heterologous genes in response to an exogenously added compound allow for conditional expression of oncogenes inserted under the control of the promoter. The addition of the promoting agent then allows stable cell lines to be expanded and transformed. When placed *in vivo*, expression of the oncogene is turned off, unless the activating factor is provided.

Examples of such systems include the lac repressor system (Fieck *et al.*, 1992; Wyborski and Short, 1991; each incorporated herein by reference), the tetracycline regulatory system (U.S. Patent 5,464,758; Gossen and Bujard, 1992; Gossen *et al.*, 1995; each incorporated herein by reference), and the ecdysone inducible expression system (No *et al.*, 1996), which is commercially available from Invitrogen, Incorporated (San Diego, CA; catalog number K1000-01). Examples of exogenously added compounds include, but are not limited to, lactose, IPTG, tetracycline or a tetracycline derivative, ecdysone or muristerone A, rapamycin and FK506.

B. Physical Separation

The present invention contemplates the use of the *Cre/Lox* site-specific recombination system (Sauer, 1993, available through Gibco/BRL, Inc., Gaithersburg, Md.) to rescue specific genes out of a genome, for example to remove one or more immortalizing constructs out of the genome. Briefly, the system involves the use of a bacterial nucleotide sequence knows as a *Lox*P site, which is recognized by the bacterial *Cre* protein. The *Cre* protein catalyzes a site-specific recombination event. This event is bidirectional, *i.e., Cre* will catalyze the insertion of sequences at a *Lox*P site or excise sequences that lie between two *Lox*P sites. Thus, if a construct for insertion also has flanking *Lox*P sites, introduction of the *Cre* protein, or a polynucleotide encoding the *Cre* protein, into the cell will catalyze the removal of the construct DNA. This technology is enabled in U.S. Patent No. 4,959,317, which is hereby incorporated by reference in its entirety.

The present invention also contemplates the use of recombination activating genes (RAG) 1 and 2 to rescue immortalizing constructs or other specific genes from the genome of immortalized cell lines. RAG-1 (GenBank accession number M29475) and RAG-2 (GenBank accession numbers M64796 and M33828) recognize specific recombination signal sequences (RSSs) and catalyze V(D)J recombination required for the assembly of immunoglobulin and T cell receptor genes (Schatz *et al.*, 1989; Oettinger *et al.*, 1990; Cumo and Oettinger, 1994). Transgenic expression of RAG-1 and RAG-2 proteins in non-lymphoid cells supports V(D)J recombination of reporter substrates (Oettinger *et al.*, 1990). For use in the present invention, the immortalizing construct of interest is engineered to contain flanking RSSs. Following immortalization, the immortalizing construct that is internal to the RSSs can be deleted from the genome by the transient expression of RAG-1 and RAG-2 in the immortalized cell.

X. Further Engineering

The present invention also contemplates augmenting or increasing the capabilities of cells to produce one or more biologically active polypeptides, and changing the pattern of response to various secretagogues, including re-engineering responsiveness to certain secretagogues, if necessary. Additional methods of engineering cells can be found in PCT patent applications publication numbers WO 97/26334 and WO 97/26321, incorporated specifically herein by reference in their entirety.

Additionally, in certain aspects of the present invention, reducing or eliminating the production of one or more endogenous proteins or peptides will be desired. Blocking expression of one or more endogenous gene product is an important modification of host cells according to the present invention. The targeted endogenous gene encodes a protein normally secreted by the host cell. Blocking expression of this endogenous gene product, while engineering high level expression of genes of interest, represents a unique way of designing cells for protein production.

Cells generated by this two-step process express heterologous proteins, including a variety of natural or engineered proteins (fusions, chimeras, protein fragments, *etc.*). Cell lines developed in this way are uniquely suited for *in vivo* cell-based delivery or *in vitro* large-scale production of defined peptide hormones with little or no contaminating or unwanted endogenous protein production.

A number of basic approaches are contemplated for blocking of expression of an endogenous gene in host cells, including homologous recombination, random integration, antisense technology, ribozyme technology and genomic site directed mutagenesis. Methods for blocking production of endogenous proteins and peptides is discussed in greater detail in the context of reduction of hexokinase in **Section XI(B)** below.

A. Amplification

To increase the output of an endogenous peptide or even of a heterologous peptide, the present invention contemplates the supplemental expression or overexpression of proteins involved in maintaining the specialized phenotype of host cells, especially their secretory capacity. Such proteins may be used to supplement the cell's natural enzymes. Engineering the overexpression of a cell type-specific transcription factor, such as the Insulin Promoter Factor 1 (IPF1) found in pancreatic β-cells (Ohlsson *et al.*, 1993), is particularly contemplated.

Insulin promoter factor 1 (IPF-1; also referred to as STF-1, IDX-1, PDX-1 and βTF-1) is a homeodomain-containing transcription factor proposed to play an important role in both pancreatic development and insulin gene expression in mature β-cells (Ohlsson *et al.*, 1993, Leonard *et al.*, 1993, Miller *et al.*, 1994, Kruse *et al.*, 1993). In embryos, IPF-1 is expressed prior to islet cell hormone gene expression and is restricted to positions within the primitive foregut where the pancreas will later form. Indeed, mice in which the IPF-1 gene is disrupted by targeted knockout do not form a pancreas (Jonsson *et al.*, 1994). Later in pancreatic development, as the different cell types of the pancreas start to emerge, IPF-1 expression becomes restricted predominantly to β-cells. IPF-1 binds to TAAT consensus motifs contained within the FLAT E and P1 elements of the insulin enhancer/promoter, whereupon, it interacts with other transcription factors to activate insulin gene transcription (Peers *et al.*, 1994).

Although IPF-1 will generally be present in the resultant immortalized human β-cells of the present invention, the overexpression of IPF-1 in human β-cell lines may be used to serve two purposes. First, it will increase transgene expression under the control of the insulin enhancer/promoter. Second, as IPF-1 appears to be critically involved in β-cell maturation, stable overexpression of IPF-1 in the β-cell lines should encourage these cells to maintain the differentiated function of a normal human β-cell.

Additional cell-specific transcription factors contemplated for use in the present invention include, but are not limited to, CDX-3 and LMX-1 (LMX1.1 and LMX1.2) (German *et al.*, 1992b; German *et al.*, 1994), NKX6.1 (Inoue *et al.*, 1997), NKX2.2 (Sussel *et al.*, 1998), Beta-2 (Sander and German, 1997), E47/Pan-1 (Johnson *et al.*, 1997), ISL1 (Ahlgren *et al.*, 1997) and PAX6 (Sander *et al.*, 1997).

B. Processing Enzymes

The augmentation and increase of the stable cells' capacity to produce biologically active polypeptides can also be accomplished by overexpressing the proteins involved in protein processing, such as the endoproteases PC2 and PC3 (Steiner *et al.*, 1992) or even the peptide amidating enzyme, PAM (Eipper *et al.*, 1992). The use of increasing the expression of other dibasic peptidases, such as furin, is also contemplated, as is the expression of carboxypeptidases.

C. Modified Secretory Response

The present invention further includes embodiments where the resultant immortalized neuroendocrine cells are further engineered to modify the secretion of the endogenous secretory polypeptide in response to one or more secretagogues.

The engineering of the resultant immortalized cells to generate a more physiologically-relevant regulated secretory response includes engineering the expression or overexpression of signaling proteins known to play a role in the regulated secretory response of neuroendocrine cells. These include cell surface proteins such as the β-cell-specific inwardly rectifying potassium channel (β-cell inward rectifier, BIR or KIR; Inagaki *et al.*, 1995), involved in release of the secretory granule contents upon glucose stimulation, the sulfonylurea receptor (SUR), and ATP sensitive channel. Other heterologous releasing factor receptors may be used in these aspects of the invention, as may adrenergic receptors and the like.

Other cell surface signaling receptors which assist with potentiating the glucose-stimulated degranulation of β-cells include the glucagon-like peptide I receptor (Thorens, 1992) and the glucose-dependent insulinotropic polypeptide receptor (also known as gastric inhibitory peptide receptor) (Usdin *et al.*, 1993), which can also be engineered into immortalized human neuroendocrine cells according to the present invention. These β-cell-specific signaling receptors, as well as GLUT-2 and glucokinase (see below), are involved in secretory granule release in response to glucose. In this way, glucose stimulated release of a peptide targeted to the secretory granule can be re-engineered or enhanced.

In still further embodiments, other cell surface signaling proteins involved in non-glucose-stimulated release of secretory granule contents can be engineered into the immortalized human neuroendocrine cells of the present invention. Examples include releasing factor receptors such as Growth Hormone Releasing Factor Receptor (Lin *et al.*, 1992) and Somatostatin or Growth Hormone Releasing Hormone Receptor (Mayo, 1992).

D. Cell Signaling Machinery

The pancreatic β-cell is continually exposed to a complex mixture of molecules that modulate insulin synthesis, storage, and exocytosis. The information in this mixture is translated to regulatory signals by three distinct mechanisms: (1) transport into the cell and metabolism of fuels, (2) ion fluxes, relative to extracellular and intracellular ion pools, and (3) hormonal signals that are mediated via receptors (reviewed in Komatsu *et al.*, 1997). The transport and metabolism of glucose is a dominant signal that regulates insulin secretion. A large portion of the glucose effect is mediated by K^+-ATP channels, depends on membrane polarity, and regulates the influx of extracellular calcium through L-type Ca^{2+} channels. Amino acids are another fuel that participate in insulin secretion via the regulation of the K^+-ATP channel.

Glucose metabolism also affects intracellular Ca^{2+} stores by mechanisms that are independent of K^+-ATP channels. This portion of glucose-regulated insulin secretion is augmented by many other molecules involved in glycemic control such as fatty acids and muscarinic receptor ligands. Binding to the muscarinic receptor by acetylcholine results in the activation of phospholipases, enzymes that catalyze the conversion of phophoinositides to inositol triphosphates (IP3) and diacylglycerol (DAG). Increased IP3 levels stimulate the release of Ca^{2+} from intracellular stores and contribute to signals for exocytosis of insulin. A central theme in Ca^{2+}-induced secretion is the activation of Ca^{2+}/calmodulin-dependent kinases that link Ca^{2+} levels to exocytosis (Ashcroft, 1994).

There is also evidence that glucose regulates insulin secretion by events that are both K^+-ATP channel-independent and Ca^{2+}-independent. This form of regulation applies most often to the augmenting effects observed for hormones that bind receptors such as GLP-1, GIP, pituitary adenylate cyclase activating peptide (PACAP), and vasoactive intestinal peptide (VIP). Receptors for these peptide hormones are typically coupled to GTP-binding proteins that regulate the membrane bound form of adenylate cyclase. Stimulation of the receptors results in increases in cyclic AMP levels and increases in the activity of protein kinase A, a potentiator of insulin secretion. Other secretory kinase effects may also be independent of the

K^+-ATP channels and intracellular Ca^{2+}. Protein kinase C is stimulated by DAG and functions to augment glucose-stimulated insulin secretion (Komatsu *et al.*, 1997).

Prentki has proposed a model for glucose metabolism that takes into account many aspects of stimulated insulin secretion (Prentki, 1996). This model categorizes stimulatory events as glycolytic and post-glycolytic and supports the view that the glucose-mediated regulation of insulin secretion cannot be fully explained by the effects of the K^+-ATP channels and increases in intracellular Ca^{2+}.

In the model proposed, pyruvate is a key intermediate metabolite and its fate provides two distinct mechanisms to stimulate secretion. Pyruvate dehydrogenase, which is stimulated by an increase in the ATP/ADP ratio resulting from glycolysis, moves the metabolism of glucose toward the citric acid cycle by the conversion of pyruvate to acetyl CoA. Carbon fluxes through the citric acid cycle boost the ATP/ADP ratio even higher, and stimulate the closure of K^+-ATP channels and the concomitant increases in intracellular Ca^{2+}.

Pyruvate is also a key metabolite in anaplerosis, the replenishment of citric acid cycle intermediates. This arm of pyruvate metabolism is initiated by the activity of pyruvate carboxylase, an enzyme that catalyzes the conversion of pyruvate to citrate. When citrate is abundant it can be transported from the mitochondria into the cytoplasm and converted to malonyl CoA, a molecule that provides a link between glucose metabolism and fatty acid metabolism. Increases in malonyl CoA promote the accumulation of fatty acid intermediates, potentiators of insulin secretion that appear to be independent of Ca^{2+} (Prentki, 1996).

XI. Glucose-Responsive Insulin Secretion

Further engineering of the resultant immortalized human neuroendocrine cells of the present invention is also contemplated. For example, the glucose-responsiveness can be re-engineered into an immortalized human neuroendocrine cell that secretes insulin but in which the glucose-response has been lost, diminished or shifted.

The basis for engineering the immortalized cells to produce a cell with glucose-regulated insulin secretion is disclosed in U.S. Patent 5,427,940, incorporated herein by reference. U.S. Patent 5,427,940 discloses islet and non-islet cell lines of neuroendocrine origin which are engineered for insulin expression and glucose regulation. First, even the insulin gene can be supplied to such an engineered cell and, although this will not be required in many aspects of the present invention, it is also contemplated.

The basis for such engineering originated in part with studies using AtT-20 cells, which are derived from ACTH secreting cells of the anterior pituitary. It has been demonstrated that stable transfection of AtT-20 cells with a construct in which a viral promoter is used to direct expression of the human proinsulin cDNA results in cell lines that secrete the correctly processed and mature insulin polypeptide (Moore *et al.*, 1983). Insulin secretion from such lines (generally termed AtT-20ins) can be stimulated by agents such as forskolin or dibutyryl cAMP, with the major secreted product in the form of mature insulin. These results suggest that these cells contain a regulated secretory pathway that is similar to that operative in the islet β-cell (Moore *et al.*, 1983, Gross *et al.*, 1989). More recently, it has become clear that the endopeptidases that process proinsulin to insulin in the islet β-cell, termed PC2 and PC3, are also expressed in AtT-20ins cells (Smeekens and Steiner, 1990, Hakes *et al.*, 1991).

A. GLUT-2 and Glucokinase

However, AtT-20ins cells do not respond to glucose as a secretagogue (Hughes *et al.*, 1991). Interestingly, AtT-20 cells express the glucokinase gene (Hughes *et al.*, 1991, Liang *et al.*, 1991) and at least in some lines, low levels of glucokinase activity (Hughes *et al.*, 1991; 1992; Quaade *et al.*, 1991), but are completely lacking in GLUT-2 expression (Hughes *et al.*, 1991; 1992). Stable transfection of these cells with GLUT-2, but not the related transporter GLUT-1, confers glucose-stimulated insulin secretion (U.S. Patent 5,427,940; Hughes *et al.*, 1992, 1993).

The studies with AtT-20ins cells are important because they demonstrate that neuroendocrine cell lines that lack glucose-stimulated peptide release may be engineered for this function. Therefore, once an immortalized human neuroendocrine cell that has a regulated secretory pathway has been generated by the present invention, certain elements of the responsiveness can be re-engineered into the stable cell. In contrast, the "regulated secretory pathway", including the secretory granules, endopeptidases and post-translational modification enzymes, cannot be re-engineered into a cell lacking such a pathway.

One of the present inventors has previously shown that GLUT-2 and glucokinase work in tandem as the "glucose sensing apparatus" of the β-cell (U.S. Patent 5,427,940). U.S. Patent 5,427,940, incorporated herein by reference, describes methods for conferring glucose sensing in neuroendocrine cells and cell lines by transfection of such cells with one or more genes selected from the insulin gene, the glucokinase gene and the GLUT-2 glucose transporter gene, so as to provide an engineered cell having all three of these genes. The glucokinase and GLUT-2 genes are thus preferred for use in re-engineering stable human cells.

U.S. Patent 5,427,940 discloses that three functional genes are required to give glucose-responsive insulin secreting capacity to a cell: an insulin gene, a GLUT-2 glucose transporter gene and a glucokinase gene. In the practice of the re-engineering aspects of the present invention, therefore, it may be that only one of these three genes needs to additionally supplied, expressed or overexpressed.

Thus, if the immortalized human cell produces and expresses a reasonable level of insulin, but in a non-regulated manner, the provision of either or both of a functional glucokinase gene and a GLUT-2 gene will be desired. One of ordinary skill in the art will be readily able to test the levels of glucokinase and GLUT-2 expression, either by molecular biological hybridization or biochemical activity assays, in order to determine which one or both of such enzymes is not sufficiently expressed or active and should therefore be supplied in recombinant form. If the stable cell does not express either of the aforementioned genes in a functional fashion, or at physiological levels, it will be preferred to introduce both genes. In re-engineering glucose-responsiveness using GLUT-2 and/or glucokinase the constructs of GenBank accession numbers J03145 and M25807, respectively, may be used. In other embodiments, even the insulin gene could be re-engineered and overexpressed in an immortalized neuroendocrine cell of the invention.

B. Hexokinase Reduction

In studies in which the stable transfection of AtT-20ins cells with GLUT-2, but not GLUT-1, conferred glucose-stimulated insulin secretion, this was achieved with maximal responsiveness at subphysiological glucose levels. One of the inventors reasoned that this was likely due to a non-optimal hexokinase:glucokinase ratio (U.S. Patent 5,427,940).

In re-engineering glucose-responsiveness, the stable cells of the invention may be modified to any degree such that they have a reduced a low K_m hexokinase activity relative to the stable parent cell from which the re-engineered cell was prepared. Depending on the intended use of the cells, cells in which a moderate hexokinase inhibition is achieved will have utility. Such inhibition levels are contemplated to be those in which the low K_m hexokinase activity is reduced by at least about 5%, about 10%, about 15%, about 20%, or about 25% relative to control levels.

Re-engineered cells exhibiting more significant inhibition are also contemplated within the invention. Accordingly, cells in which the low K_m hexokinase activity is reduced by about 30%, about 40%, about 50%, about 60% or even about 70% or higher, with respect to control levels, are contemplated as part of this invention and will be preferred in certain embodiments.

In embodiments of re-engineering an immortalized cell to secrete insulin in response to glucose, other parameters may be applied in assessing useful levels of low K_m hexokinase inhibition. For example, it may be desired to determine the ratio of glucokinase to hexokinase (GK:HK ratio) and to monitor changes in this ratio as hexokinase is inhibited. It will be understood that a cell in which this ratio is changed to reflect the ratio commonly observed in functional or natural pancreatic β-cells, or in which the ratio is changed towards this, will be an advantageous engineered cell in the context of this invention.

In certain preferred embodiments, it is contemplated that cells of this invention will have a low K_m hexokinase activity that has been reduced to a level appropriate to confer more physiological insulin secretion capacity to the cell. This includes re-engineered cells that have a near-homeostatic insulin secretion capacity.

"Engineered cells that exhibit more physiological insulin secretion" are cells that exhibit glucose-stimulated insulin secretion (GSIS) closer to the normal range than the parent stable cell from which they were prepared. In this regard, the maximal glucose response of previously described cell lines generally occurs at subphysiological glucose concentrations of between about 10 μM and about 100 μM.

The GSIS of normal islet β-cells generally occurs at glucose concentrations of between about 3 mM to 20 mM, with ranges of 5 to 20 mM and 4 to 9 mM being frequently reported. Insulin responses in these ranges would therefore be described as "near-homeostatic insulin secretion." Cells that comprise an inhibitor in an amount effective to reduce the low K_m hexokinase activity of the cell to a level sufficient to confer insulin secretion in response to an extracellular glucose concentration of between about 1 mM and about 20 mM will thus be most preferred. Extracellular glucose concentrations of "between about 1 mM and about 20 mM " will be understood to include each and every numerical value within this range, such as being about 1, 2, 3, 4, 5, 6, 7, 7.5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and about 20 mM or so.

To re-engineer the ratios of glucokinase to hexokinase by inhibiting hexokinase, and thus to render the glucose-responsive insulin secretion more physiological, any one of a variety of methods may be employed, including blocking of expression of the gene in the immortalized human cells and/or inhibiting or reducing the activity of any protein produced. In creating molecular biological tools to effect these methods, the hexokinase gene construct of GenBank accession number J04526 may be utilized.

In molecular approaches suitable for reducing hexokinase activity *via* inhibiting gene expression, constructs can be designed to introduce nucleic acids complementary to a target endogenous gene, *i.e.*, an antisense approach. Expression of RNAs corresponding to these complementary nucleic acids will interfere with the transcription and/or translation of the target sequences. Inhibitory constructs can still further be designed to homologously recombine into the hexokinase endogenous gene locus, rendering the endogenous gene nonfunctional, *i.e.*, a knock-out approach. Genetic constructs may also be designed to introduce nucleic acids encoding ribozymes, RNA-cleaving enzymes, that will specifically cleave the target hexokinase mRNA.

1. Antisense

Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide, in this case, hexokinase, and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNAs, may be employed to inhibit hexokinase gene transcription or translation or both within the immortalized human neuroendocrine cells of the present invention.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a hexokinase gene. It is contemplated that effective antisense constructs will often include regions complementary to intron/exon splice junctions. Thus, antisense constructs with complementarity to regions within 50-200 bases of an intron-exon splice junction of hexokinase are contemplated for use herewith. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether the expression of hexokinase and/or other genes having complementary sequences is affected.

"Antisense" or "complementary" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

2. Homologous Recombination

Another approach for inhibiting hexokinase involves the use of homologous recombination, or "knock-out technology". Homologous recombination relies, like antisense, on the tendency of nucleic acids to base pair with complementary sequences. In this instance, the base pairing serves to facilitate the interaction of two separate nucleic acid molecules so that strand breakage and repair can take place. In other words, the "homologous" aspect of the method relies on sequence homology to bring two complementary sequences into close proximity, while the "recombination" aspect provides for one complementary sequence to replace the other by virtue of the breaking of certain bonds and the formation of others.

Put into practice, homologous recombination is used as follows. First, the target gene is selected within the host cell, in this case, hexokinase. Sequences homologous to the hexokinase target gene are then included in a genetic construct, along with some mutation that will render the target gene inactive (stop codon, interruption, and the like). The homologous sequences flanking the inactivating mutation are said to "flank" the mutation. Flanking, in this context, simply means that target homologous sequences are located both upstream (5′) and downstream (3′) of the mutation. These sequences should correspond to some sequences upstream and downstream of the target gene. The construct is then introduced into the cell, thus permitting recombination between the cellular sequences and the construct.

As a practical matter, the genetic construct will normally act as far more than a vehicle to interrupt the gene. For example, it is important to be able to select for recombinants and, therefore, it is common to include within the construct a selectable marker gene. This gene permits selection of cells that have integrated the construct into their genomic DNA by conferring resistance to various biostatic and biocidal drugs. In addition, a heterologous gene that is to be expressed in the cell also may advantageously be included within the construct. The arrangement might be as follows:

> ...vector•5′-flanking sequence•heterologous gene• selectable marker gene•flanking sequence-3′•vector...

Thus, using this kind of construct, it is possible, in a single recombinatorial event, to (i) "knock out" an endogenous gene, (ii) provide a selectable marker for identifying such an event and (iii) introduce a heterologous gene for expression.

Another refinement of the homologous recombination approach involves the use of a "negative" selectable marker. One example is the use of the cytosine deaminase gene in a negative selection method as described in U.S. Patent No. 5,624,830 (incorporated herein by reference). This marker, unlike the selectable marker, causes death of cells which express the marker. Thus, it is used to identify undesirable recombination events. When seeking to select homologous recombinants using a selectable marker, it is difficult in the initial screening step to identify proper homologous recombinants from recombinants generated from random, non-sequence specific events. These recombinants also may contain the selectable marker gene and may express the heterologous protein of interest, but will, in all likelihood, not have the desired "knock out" phenotype. By attaching a negative selectable marker to the construct, but outside of the flanking regions, one can select against many random recombination events that will incorporate the negative selectable marker. Homologous recombination should not introduce the negative selectable marker, as it is outside of the flanking sequences.

3. Random Integration

Though lacking the specificity of homologous recombination, there may be situations where random integration will be used as a method of knocking out a particular endogenous gene. Unlike homologous recombination, the recombinatorial event here is completely random, *i.e.*, not reliant upon base-pairing of complementary nucleic acid sequences. Random integration is like homologous recombination, however, in that a gene construct, often containing a heterologous gene and a selectable marker, integrates into the target cell genomic DNA *via* strand breakage and reformation.

Because of the lack of sequence specificity, the chances of any given recombinant integrating into the target gene are greatly reduced. Also possible is integration into a second loci, resulting in the loss of expression of the gene of interest. This second locus could encode a transcription factor needed for expression of the first gene, a locus control region needed for the expression of the first gene, *etc.* As a result, it may be necessary to "brute force" the selection process. In other words, it may be necessary to screen hundreds of thousands of drug-resistant recombinants before a desired mutant is found. Screening can be facilitated, for example, by examining recombinants for expression of the target gene using immunologic or even functional tests; expression of the target gene indicate recombination elsewhere and, thus, lack of suitability.

4. Ribozymes

Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U.S. Patent No. 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990; Sioud *et al.*, 1992). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

Several different ribozyme motifs have been described with RNA cleavage activity (Symons, 1992). Examples that are expected to function equivalently for the down regulation of low K_m hexokinases include sequences from the Group I self splicing introns including Tobacco Ringspot Virus (Prody *et al.*, 1986), Avocado Sunblotch Viroid (Palukaitis *et al.*, 1979 and Symons, 1981), and Lucerne Transient Streak Virus (Forster and Symons, 1987). Sequences from these and related viruses are referred to as hammerhead ribozyme based on a predicted folded secondary structure.

Other suitable ribozymes include sequences from RNase P with RNA cleavage activity (Yuan *et al.*, 1992, Yuan and Altman, 1994, and U.S. Patent Nos. 5,168,053 and 5,624,824), hairpin ribozyme structures (Berzal-Herranz *et al.*, 1992 and Chowrira *et al.*, 1993) and Hepatitis Delta virus based ribozymes. The general design and optimization of ribozyme directed RNA cleavage activity has been discussed in detail (Haseloff and Gerlach, 1988, Symons, 1992, Chowrira *et al.*, 1994, and Thompson *et al.*, 1995).

The other variable on ribozyme design is the selection of a cleavage site on a given target RNA. Ribozymes are targeted to a given sequence by virtue of annealing to a site by complimentary base pair interactions. Two stretches of homology are required for this targeting. These stretches of homologous sequences flank the catalytic ribozyme structure defined above. Each stretch of homologous sequence can vary in length from 7 to 15 nucleotides. The only requirement for defining the homologous sequences is that, on the target RNA, they are separated by a specific sequence which is the cleavage site. For hammerhead ribozyme, the cleavage site is a dinucleotide sequence on the target RNA is a uracil (U) followed by either an adenine, cytosine or uracil (A,C or U) (Perriman *et al.*, 1992 and Thompson *et al.*, 1995). The frequency of this dinucleotide occurring in any given RNA is statistically 3 out of 16. Therefore, for a given target messenger RNA of 1000 bases, 187 dinucleotide cleavage sites are statistically possible. The message for low K_m hexokinases targeted here are greater than 3500 bases long, with greater than 500 possible cleavage sites.

The large number of possible cleavage sites in the low K_m hexokinases coupled with the growing number of sequences with demonstrated catalytic RNA cleavage activity indicates that a large number of ribozymes that have the potential to downregulate the low K_m hexokinases are available. Designing and testing ribozymes for efficient cleavage of a target RNA is a process well known to those skilled in the art. Examples of scientific methods for designing and testing ribozymes are described by Chowrira *et al.*, (1994) and Lieber and Strauss (1995), each incorporated by reference. The identification of operative and preferred sequences for use in hexokinase-targeted ribozymes is simply a matter of preparing and testing a given sequence, and is a routinely practiced "screening" method known to those of skill in the art.

In terms of inhibiting protein activity, hexokinase may further be inhibited by any one or more of the methods described below. Particularly contemplated are agents that displace hexokinase from mitochondria and thus competitively reduce hexokinase activity. This group of agents includes inactive hexokinases and fragments thereof that retain mitochondrial binding functions and, also, hexokinase-glucokinase chimeras that further substitute glucokinase activity for hexokinase activity. Metabolic inhibitors of hexokinase activity that stimulate the production of trehalose-6-phosphate may also be used. All of the inhibitory agents may be provided in the form of a gene or vector that expresses the particular agent.

5. Genomic Site-Directed Mutagenesis

Through analysis of radiation-sensitive mutants of *Ustilago maydis*, genes have been characterized that participate in DNA repair (Tsukuda *et al.*, 1989; Bauchwitz and Holloman, 1990). One such gene, *REC2*, encodes a protein that catalyzes homologous pairing between complementary nucleic acids and is required for a functional recombinational repair pathway (Kmiec *et al.*, 1994; Rubin *et al.*, 1994). *In vitro* characterization of the REC2 protein showed that homologous pairing was more efficient between RNA-DNA hybrids than the corresponding DNA duplexes (Kmiec *et al.*, 1994; PCT, WO 96/22364). However, efficiency in pairing between DNA:DNA duplexes could be enhanced by increasing the length of the DNA oligonucleotides (Kmiec *et al.*, 1994).

These observations led investigators to test the use of chimeric RNA-DNA oligonucleotides (RDOs) in the targeted modification of genes in mammalian cell lines (Yoon *et al.*, 1996; Cole-Strauss *et al.*, 1996; PCT WO95/15972). The RNA-DNA oligonucleotides that were used to test this application contained self-annealing sequences such that double-hairpin capped ends are formed. This feature is thought to increase the *in vivo* half-life of the RDO by decreasing degradation by helicases and exonucleases. Further, the RDOs contained a single base pair that differs from the target sequence and otherwise aligns in perfect register. It is believed that the single mismatch will be recognized the DNA repair enzymes. And the RDOs contained RNA residues modified by 2'-O-methylation of the ribose sugar. Such modification makes the RDO resistant to degradation by ribonuclease activity (Monia *et al.*, 1993).

Two separate experimental systems have been used to test the use of RDOs for targeted gene disruption in mammalian cell lines. In one system RDOs were used to target and correct an alkaline phosphatase cDNA in that was maintained in the episomal DNA of Chinese hamster ovary cells. An inactive form of alkaline phosphatase was converted to a wild-type form with an efficiency of about 30% (Yoon *et al.*, 1996). In a second system, a genetic mutation within chromosomal DNA was targeted and corrected. A lymphoid blast cell line was derived from a patient with sickle cell disease who was homozygous for a point mutation in the β–globin gene. Here again the overall frequency of gene conversion from the mutant to the wild-type form was very high and was found to be dose-dependent on the concentration of the RDOs (Cole-Strauss *et al.*, 1996).

If the use of RDOs or DNA oligonucleotides for the purposes of targeted gene conversion is broadly applicable to various mammalian cell lines, then it offers several advantages to current technologies that have been used to accomplish gene disruption such as homologous recombination. First, if gene conversion by RDO or DNA oligonucleotides occurs in various cell lines at an efficiency of 30% then this will represent a much higher rate than has been reported for targeted gene disruption *via* homologous recombination. Secondly, only short sequences are required for gene disruption by RDOs or DNA oligonucleotides (typically 60 mers to 70 mers); whereas homologous recombination requires very long stretches of complementary sequences. Homologous sequences from 9 to 15 kilobases are typically recommended in the construction of targeting vectors. As a result, construction of DNA vectors for homologous recombination usually involves extensive gene mapping studies and time consuming efforts in the isolation of genomic DNA sequences. Such efforts are unnecessary if RDOs are used for targeted gene conversions. Thirdly, assays for gene conversion by RDOs can be performed 4 to 6 hours following introduction of the RDOs or DNA oligonucleotides into the cell. In contrast, gene conversion by homologous recombination requires a relatively long period of time (days to weeks) between the time of introducing the targeting vector DNA and assaying for recombinants.

6. Mitochondrial Binding

Low K_m hexokinases are distinguished from glucokinase in that they are allosterically regulated by glucose-6-phosphate and by binding to mitochondria (Wilson, 1968; 1973; 1985; 1994). Micromolar concentrations of glucose-6-phosphate inhibit the activities of hexokinases I, II, and III, but appreciable inhibition of glucokinase requires glucose-6-phosphate concentrations in excess of 10 mM. Binding of hexokinases I and II to mitochondria alters their kinetic properties (Wilson, 1968; 1985; 1995), while glucokinase does not appear to be capable of binding to mitochondria at all (Becker *et al.* 1996).

When bound to mitochondria, hexokinase I undergoes an increase in affinity (a decrease in K_m) for its substrate ATP (Wilson, 1985). In addition, the enzyme becomes far less inhibitable by glucose-6-phosphate, as indicated by a several-fold increase in K_i for this ligand (Wilson, 1985). Studies with hexokinase I have revealed the existence of two types of mitochondrial binding sites (Kabir and Wilson, 1994). Glucose-6-phosphate causes displacement of a proportion of mitochondrially-bound hexokinase from one type of site. The enzyme that remains bound to mitochondria after glucose-6-phosphate treatment is considered to occupy the second site, from which it can be removed by treatment with 0.5 M KSCN.

It is known that limited digestion of hexokinase I with chymotrypsin yields an enzyme fragment that retains catalytic activity but that loses its capacity for mitochondrial binding, and that enzyme treated in this manner is lacking in a portion of its N-terminal domain (Polakis and Wilson, 1985). The N-terminal sequences of both hexokinases I and II are relatively hydrophobic, and it has been shown that the hydrophobic N-terminus of hexokinase I is capable of insertion into the lipid bilayer of the mitochondrial membrane (Xie and Wilson, 1988).

Subsequently, Gelb *et al.*, (1992) demonstrated that a chimeric protein consisting of the N-terminal 15 amino acids of hexokinase I fused to chloramphenicol acetyltransferase was capable of binding to rat liver mitochondria, and that this binding was competitive with authentic hexokinase I (Gelb *et al.* 1992). While the results of Gelb *et al.* (1992) argue for the importance of this small N-terminal segment in targeting of hexokinase to mitochondria, others have suggested that other regions of the molecule may also be important in stabilizing the interaction (Polakis and Wilson, 1985; Felgner *et al.*, 1977; Smith *et al.*, 1991). This is based on studies showing that hexokinase I binding to mitochondria is stabilized by Mg^{2+}, an effect likely reflecting electrostatic interactions between the enzyme and the outer mitochondrial membrane (*i.e.*, not involving the N-terminal 15 amino acids that are intercalated into the membrane).

Further aspects of the present invention concern the inhibition of hexokinase based upon the displacement of hexokinase from mitochondria. The inventors term this the "dominant:negative strategy". The inhibitory agents for use in these aspects of the invention will be agents that competitively reduce low K_m hexokinase activity. One example of such an agent is the glucokinase enzyme. Other possible examples include glycerol kinase. Preferred examples of these agents are agents that lack low K_m hexokinase activity and that displace low K_m hexokinase from mitochondria, as described below.

One group of hexokinase inhibitors of this type are those that compete for hexokinase binding sites and consequentially displace endogenous low K_m hexokinases from their mitochondrial binding sites within an intact cell. Such "displacing agents" will generally comprise a mitochondrial binding region from the N-terminal domain of a low K_m hexokinase, such as hexokinase I or hexokinase II.

Compositions for use in these aspects may comprise the N-terminal 15 amino acids of a hexokinase enzyme, preferably hexokinase I or II, since this segment should be easily expressed in cells and retained as a stable peptide. Constructs comprising the entire N-terminal domain of either hexokinase I or hexokinase II, or the intact, full-length hexokinase I or II proteins that have been rendered inactive by site-directed mutagenesis of amino acids that are important for the enzyme's catalytic function are also contemplated. Constructs based upon hexokinase I will be particularly, or even exclusively, preferred in certain embodiments.

The reason for preferring the N-terminal domain construct is that this element seems to comprise a complete structural domain, based upon studies in which this domain can be expressed in bacteria and shown to bind glucose-6-phosphate (Wilson, 1994; Arora *et al.*, 1993; White *et al.*, 1987; White *et al.*, 1990). This suggests that the intact N-terminal domain should fold and form a structure analogous to its structure in the full-length hexokinase I or II protein. As the inventors contemplate that this structure mediates attachment of the intact hexokinase protein to mitochondria, the intact, correctly folded N-terminal domain is a preferred embodiment of hexokinase inhibition.

For embodiments involving the N-terminal domain, a segment comprising amino acids 1-455 is preferred because of a naturally occurring *Nco*I restriction enzyme site in the DNA sequence corresponding to amino acid 482. This *Nco*I site allows the fragment encoding the N-terminal domain to be easily isolated and subcloned, and also allows direct fusion of the N-terminal domain of hexokinase to the intact functional sequence of glucokinase *via* an *Nco*I site located at the AUG start codon of this gene.

Of course, it will be understood that peptides, polypeptides and protein domains of any intermediate length between about 15 amino acids and about 455 amino acids, and longer proteins, may be used in displacing endogenous hexokinase from the mitochondria. Accordingly, constructs comprising about 20, about 50, about 100, about 150, about 200, about 300 or about 400 amino acids in length may be used for these purposes.

It is also contemplated that an intact hexokinase protein that is rendered catalytically inactive will interact with mitochondria in a manner identical to the active proteins. Expression of such a HK variant is therefore another method for inhibiting endogenous HK (Baijal *et al.*, 1992). Inactivated, hexokinase proteins include those that have been subjected to chemical mutagenesis and also those produced using molecular biological techniques and recombinant protein production.

The identification of appropriate polypeptide regions and/or particular amino acid sites that may be targeted in order to inactivate hexokinase will be known to those of skill in the art.

The crystal structure of certain hexokinase enzymes is available. Coupling the crystal structure information with a comparison of the primary sequence information for various distinct hexokinases will allow one to identify those regions and sites that are important for hexokinase activity, such as the binding sites for ATP, glucose and glucose-6-phosphate. This information can be used in connection with preparing mutants and variants for use herewith. Deletion of certain amino acids or peptide segments, as may be achieved by molecular biological manipulation, is another contemplated method for preparing inactive hexokinases.

At least part of hexokinase binding to mitochondria is *via* interactions with members of a family of proteins known as voltage-dependent anion channels (VDAC) or porins (Fiek *et al.*, 1982; Linden *et al.*, 1982). These porins form a channel through which metabolites such as ATP and various anions traverse the outer mitochondrial membrane. Binding of hexokinases to porin thus may ensure a supply of intramitochondrially-generated ATP as substrate.

The enzyme glycerol kinase is another protein thought to bind to mitochondria *via* porins or VDACs (Adams *et al.*, 1991). Glycerol kinase catalyzes formation of glycerol phosphate from glycerol, using ATP as phosphate donor. Thus, expression of glycerol kinase in cell lines represents another means by which to displace endogenous low-K_m hexokinases from their normal mitochondrial binding site.

A particularly powerful method of inhibiting hexokinase within a mammalian cell involves the displacement of hexokinase from the mitochondria and the concomitant provision of inactive glucokinase. This is advantageously achieved by providing to the cell a hexokinase-glucokinase chimera or fusion protein, in which the hexokinase portion is capable of binding to the mitochondria and yet does not exhibit hexokinase catalytic activity, and in which the glucokinase portion is catalytically active. Chemically-fused polypeptides are a possibility, but recombinant proteins are naturally most preferred for use in this manner. The identification of appropriate hexokinase fragments for use in such a chimera has been described herein above.

In terms of the glucokinase portions of these fusion proteins, any glucokinase-derived sequence that contains enough primary sequence information to confer glucokinase catalytic activity to the chimera will be useful in this context. However, it will often be preferred to use the entire glucokinase enzyme as this is more straightforward in terms of methodology. Again, one may look to the extensive information available in various published references in order to assist with the identification of appropriate glucokinase enzymes or fragments thereof.

7. Trehalose-6-Phosphate Metabolism

In Baker's yeast, glucose phosphorylation is also catalyzed by a family of hexokinases that are related in sequence and function to the mammalian hexokinase gene family. Yeast cells, however, contain other genes involved in carbohydrate metabolism for which there are no mammalian counterparts. The trehalose-6-phosphate synthase/trehalose-6-phosphate phosphatase complex is an example of such an activity.

The trehalose-6-phosphate synthase/phosphatase complex catalyzes the formation of trehalose, a disaccharide of two glucose molecules (α-D-glucopyranosyl (1-1) α-D-glucopyranoside) by first forming trehalose-6-phosphate by condensation of two molecules of glucose-6-phosphate and then using its phosphatase activity to remove the phosphate groups to generate free trehalose (Bell *et al.*, 1992). Trehalose is thought to represent a form of storage polysaccharide in yeast, bacteria and other lower organisms, but neither the trehalose-6-phosphate synthase enzyme complex nor its products trehalose-6-phosphate or free trehalose are known to be present in mammalian cells.

Blazquez *et al.* have demonstrated that trehalose-6-phosphate can inhibit the activity of hexokinases from a variety of different organisms, including rat brain, which expresses predominantly hexokinase I (Blazquez *et al.*, 1993). This has led to the suggestion that trehalose-6-phosphate may be an important regulator of glycolytic flux in yeast cells. Consistent with this notion, the yeast gene known as *cif-1* was originally cloned from yeast that are unable to grow in glucose (Blazquez *et al.*, 1993) and subsequently shown to be identical to the smallest subunit (56 kDa) of the trehalose phosphate synthase/trehalose-6-phosphate phosphatase complex (Bell *et al.*, 1992). Cells lacking in the CIF-1 gene product exhibit rapid depletion of ATP, presumably because they are unable to produce trehalose-6-phosphate that normally serves to moderate yeast hexokinase activity. It is believed that the 56 kDa CIF-1 gene product encodes the trehalose phosphate synthase activity (Bell *et al.*, 1992).

A further method for inhibiting low K_m hexokinase activity in mammalian cells is to express an enzyme, such as yeast trehalose-6-phosphate synthase, that will allow trehalose-6-phosphate to accumulate. This will have two effects. First, the accumulated trehalose-6-phosphate will serve to allosterically inhibit endogenous low K_m hexokinase activity. Second, where trehalose-phosphate synthase is used, this enzyme will divert glucose-6-phosphate into trehalose-6-phosphate at low, non-stimulatory glucose concentrations where low K_m hexokinases but not glucokinases are active, thereby "short-circuiting" metabolic signaling for insulin secretion, which is thought to require ATP produced *via* further glucose metabolism (Newgard and McGarry, 1995).

A currently preferred gene for use in these aspects is the *S. cerevisiae* gene encoding trehalose-6-phosphate synthase (TPS1). Genes from several other organisms encoding trehalose-6-phosphate synthase have been isolated and the amino acid sequences deduced. These include *E. coli* (Accession # X69160), *S. pombe* (# Z29971), *Mycobacterium laprae* (# U15187) and *Aspergillus niger* (# U07184). It is contemplated that any of the foregoing or other biological functional equivalents thereof may be used in the context to the present invention.

8. Combined Inhibitory Methods

Any of the methods of HK inhibition described above may be combined with one another and, particularly, may also be used in combination with glucokinase overproduction. Glucokinase overproduction alone is even thought to be a useful method of inhibiting hexokinase, as set forth below.

Low K_m hexokinases, including hexokinases I and II that are present at high levels in mammalian cell lines, are inhibited by glucose-6-phosphate. Thus, methods for maintaining glucose-6-phosphate at high levels in cell lines may also be employed. The preferred method for achieving consistently high levels of glucose-6-phosphate in cells is to overexpress glucokinase in such lines.

Expression of glucokinase is considered advantageous for two distinct reasons. First, as described above and in U.S. Patent 5,427,940, expression of glucokinase is part of the basic glucose-stimulated insulin secretion re-engineering. Glucokinase expression has the added benefit of maintaining high levels of glucose-6-phosphate to keep low K_m hexokinases in an inhibited state. This advantage would become particularly relevant at glucose concentrations in the physiological range (4-9 mM), because glucokinase is active at these levels. Also, while glucokinase is a member of the hexokinase gene family, it is not itself inhibited by glucose-6-phosphate.

9. Advantages of Hexokinase Inhibition

A further implication of inhibiting low K_m hexokinase concerns the use of the re-engineered stable cells for *in vivo* therapies. It is envisioned that cell-based delivery will be conducted by maintenance of the cells *in vivo* in a perm-selective device. It is contemplated that cells with reduced levels of low K_m hexokinase activity will survive for longer periods of time in devices or capsules as a consequence of their reduced growth rates.

XII. *In vitro* Uses

A. Bioreactors

The ability to produce biologically active polypeptides is increasingly important to the pharmaceutical industry. Over the last decade, advances in biotechnology have led to the production of important proteins and factors from bacteria, yeast, insect cells and from mammalian cell culture. Mammalian cultures have advantages over cultures derived from the less advanced lifeforms in their ability to post-translationally process complex protein structures such as disulfide-dependent folding and glycosylation. Neuroendocrine cell types have added unique capacities of endoproteolytic cleaving, C-terminal amidation and regulated secretion. Indeed, mammalian cell culture is now the preferred source of a number of important proteins for use in human and animal medicine, especially those which are relatively large, complex or glycosylated.

Development of mammalian cell culture for production of pharmaceuticals has been greatly aided by the development in molecular biology of techniques for design and construction of vector systems highly efficient in mammalian cell cultures, a battery of useful selection markers, gene amplification schemes and a more comprehensive understanding of the biochemical and cellular mechanisms involved in procuring the final biologically-active molecule from the introduced vector.

However, the traditional selection of cell types for expressing heterologous proteins has generally been limited to the more "common" cell types such as CHO cells, BHK cells, C127 cells and myeloma cells. In many cases, these cell types were selected because there was a great deal of preexisting literature on the cell type or the cell was simply being carried in the laboratory at the time the effort was made to express a peptide product. Frequently, factors which affect the downstream (*e.g.,* beyond the T-75 flask) side of manufacturing scale-up were not considered before selecting the cell line as the host for the expression system.

Aspects of the present invention take advantage of the biochemical and cellular capacities of stable neuroendocrine cells as well as of recently available bioreactor technology. Growing cells according to the present invention in a bioreactor allows for large scale production and secretion of complex, fully biologically-active polypeptides into the growth media. In particular embodiments, by designing a defined media with low contents of complex proteins and using a scheme of timed-stimulation of the secretion into the media for increased titer, the purification strategy can be greatly simplified, thus lowering production cost.

1. Anchorage-dependent versus non-anchorage-dependent cultures.

Animal and human cells can be propagated *in vitro* in two modes: as non-anchorage dependent cells growing freely in suspension throughout the bulk of the culture; or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. Large scale suspension culture based on microbial (bacterial and yeast) fermentation technology has clear advantages for the manufacturing of mammalian cell products. The processes are relatively straightforward to operate and scale up. Homogeneous conditions can be provided in the reactor which allows for precise monitoring and control of temperature, dissolved oxygen, and pH, and ensure that representative samples of the culture can be taken.

However, suspension cultured cells cannot always be used in the production of biologicals. Suspension cultures are still considered to have tumorigenic potential and thus their use as substrates for production put limits on the use of the resulting products in human and veterinary applications (Petricciani, 1985; Larsson and Litwin, 1987). Viruses propagated in suspension cultures as opposed to anchorage-dependent cultures can sometimes cause rapid changes in viral markers, leading to reduced immunogenicity (Bahnemann *et al.*, 1980). Finally, sometimes even recombinant cell lines can secrete considerably higher amounts of products when propagated as anchorage-dependent cultures as compared with the same cell line in suspension (Nilsson and Mosbach, 1987). For these reasons, different types of anchorage-dependent cells are used extensively in the production of different biological products.

The current invention includes stable cells which are anchorage-dependent of nature. Anchorage-dependent cells, when grown in suspension, will attach to each other and grow in clumps, eventually suffocating cells in the inner core of each clump as they reach a size that leaves the core cells unsustainable by the culture conditions. Therefore, an efficient means of large-scale culture of anchorage-dependent cells is also provided in order to effectively take advantage of stable cells' capacity to secrete heterologous proteins.

2. Reactors and processes for suspension.

Large scale suspension culture of mammalian cultures in stirred tanks has been undertaken. The instrumentation and controls for bioreactors have been adapted, along with the design of the fermentors, from related microbial applications. However, acknowledging the increased demand for contamination control in the slower growing mammalian cultures, improved aseptic designs have been implemented, improving dependability of these reactors. Instrumentation and controls include agitation, temperature, dissolved oxygen, and pH controls. More advanced probes and autoanalyzers for on-line and off-line measurements of turbidity (a function of particles present), capacitance (a function of viable cells present), glucose/lactate, carbonate/bicarbonate and carbon dioxide are also available. Maximum cell densities obtainable in suspension cultures are relatively low at about 2-4 × 10^6 cells/ml of medium (which is less than 1 mg dry cell weight per ml), well below the numbers achieved in microbial fermentation.

Two suspension culture reactor designs are most widely used in the industry due to their simplicity and robustness of operation - the stirred reactor and the airlift reactor. The stirred reactor design has successfully been used on a scale of 8000 liter capacity for the production of interferon (Phillips *et al.*, 1985; Mizrahi, 1983). Cells are grown in a stainless steel tank with a height-to-diameter ratio of 1:1 to 3:1. The culture is usually mixed with one or more agitators, based on bladed disks or marine propeller patterns. Agitator systems offering less shear forces than blades have been described. Agitation may be driven either directly or indirectly by magnetically coupled drives. Indirect drives reduce the risk of microbial contamination through seals on stirrer shafts.

The airlift reactor, also initially described for microbial fermentation and later adapted for mammalian culture, relies on a gas stream to both mix and oxygenate the culture. The gas stream enters a riser section of the reactor and drives circulation. Gas disengages at the culture surface, causing denser liquid free of gas bubbles to travel downward in the downcomer section of the reactor. The main advantage of this design is the simplicity and lack of need for mechanical mixing. Typically, the height-to-diameter ratio is 10:1. The airlift reactor scales up relatively readily, has good mass transfer of gasses and generates relatively low shear forces.

Most large-scale suspension cultures are operated as batch or fed-batch processes because they are the most straightforward to operate and scale up. However, continuous processes based on chemostat or perfusion principles are available.

A batch process is a closed system in which a typical growth profile is seen. A lag phase is followed by exponential, stationary and decline phases. In such a system, the environment is continuously changing as nutrients are depleted and metabolites accumulate. This makes analysis of factors influencing cell growth and productivity, and hence optimization of the process, a complex task. Productivity of a batch process may be increased by controlled feeding of key nutrients to prolong the growth cycle. Such a fed-batch process is still a closed system because cells, products and waste products are not removed.

In what is still a closed system, perfusion of fresh medium through the culture can be achieved by retaining the cells with a fine mesh spin filter and spinning to prevent clogging. Spin filter cultures can produce cell densities of approximately 5×10^7 cells/ml. A true open system and the most basic perfusion process is the chemostat in which there is an inflow of medium and an outflow of cells and products. Culture medium is fed to the reactor at a predetermined and constant rate which maintains the dilution rate of the culture at a value less than the maximum specific growth rate of the cells (to prevent washout of the cell mass from the reactor). Culture fluid containing cells, cell products and byproducts is removed at the same rate. These perfused systems are not in commercial use for production from mammalian cell culture.

3. Non-perfused attachment systems.

Traditionally, anchorage-dependent cell cultures are propagated on the bottom of small glass or plastic vessels. The restricted surface-to-volume ratio offered by classical and traditional techniques, suitable for the laboratory scale, has created a bottleneck in the production of cells and cell products on a large scale. To provide systems that offer large accessible surfaces for cell growth in small culture volume, a number of techniques have been proposed: the roller bottle system, the stack plates propagator, the spiral film bottles, the hollow fiber system, the packed bed, the plate exchanger system, and the membrane tubing reel. Since these systems are non-homogeneous in their nature, and are sometimes based on multiple processes, they can sometimes have limited potential for scale-up, difficulties in taking cell samples, limited potential for measuring and controlling the system and difficulty in maintaining homogeneous environmental conditions throughout the culture.

A commonly used process of these systems is the roller bottle. Being little more than a large, differently shaped T-flask, simplicity of the system makes it very dependable and, hence, attractive. Fully automated robots are available that can handle thousands of roller bottles per day, thus eliminating the risk of contamination and inconsistency associated with the otherwise required intense human handling. With frequent media changes, roller bottle cultures can achieve cell densities of close to 0.5×10^6 cells/cm^2 (corresponding to 10^9 cells/bottle or 10^7 cells/ml of culture media).

4. Cultures on microcarriers

van Wezel (1967) developed the concept of the microcarrier culturing systems. In this system, cells are propagated on the surface of small solid particles suspended in the growth medium by slow agitation. Cells attach to the microcarriers and grow gradually to confluency of the microcarrier surface. In fact, this large scale culture system upgrades the attachment dependent culture from a single disc process to a unit process in which both monolayer and suspension culture have been brought together. Thus, combining the necessary surface for the cells to grow with the advantages of the homogeneous suspension culture increases production.

The advantages of microcarrier cultures over most other anchorage-dependent, large-scale cultivation methods are several fold. First, microcarrier cultures offer a high surface-to-volume ratio (variable by changing the carrier concentration) which leads to high cell density yields and a potential for obtaining highly concentrated cell products. Cell yields are up to 1-2 $\times 10^7$ cells/ml when cultures are propagated in a perfused reactor mode. Second, cells can be propagated in one unit process vessels instead of using many small low-productivity vessels (*i.e.*, flasks or dishes). This results in far better utilization and a considerable saving of culture medium. Moreover, propagation in a single reactor leads to reduction in need for facility space and in the number of handling steps required per cell, thus reducing labor cost and risk of contamination.

Third, the well-mixed and homogeneous microcarrier suspension culture makes it possible to monitor and control environmental conditions (*e.g.*, pH, pO_2, and concentration of medium components), thus leading to more reproducible cell propagation and product recovery. Fourth, it is possible to take a representative sample for microscopic observation, chemical testing, or enumeration. Fifth, since microcarriers settle out of suspension easily, use of a fed-batch process or harvesting of cells can be done relatively easily. Sixth, the mode of the anchorage-dependent culture propagation on the microcarriers makes it possible to use this system for other cellular manipulations, such as cell transfer without the use of proteolytic enzymes, cocultivation of cells, transplantation into animals, and perfusion of the culture using decanters, columns, fluidized beds, or hollow fibers for microcarrier retainment. Seventh, microcarrier cultures are relatively easily scaled up using conventional equipment used for cultivation of microbial and animal cells in suspension.

5. Microencapsulation of mammalian cells

One method which has shown to be particularly useful for culturing mammalian cells is microencapsulation. The mammalian cells are retained inside a semipermeable hydrogel membrane. A porous membrane is formed around the cells permitting the exchange of nutrients, gases, and metabolic products with the bulk medium surrounding the capsule. Several methods have been developed that are gentle, rapid and non-toxic and where the resulting membrane is sufficiently porous and strong to sustain the growing cell mass throughout the term of the culture. These methods are all based on soluble alginate gelled by droplet contact with a calcium-containing solution. Lim (1982) describes cells concentrated in an approximately 1% solution of sodium alginate which are forced through a small orifice, forming droplets, and breaking free into an approximately 1% calcium chloride solution. The droplets are then cast in a layer of polyamino acid that ionically bonds to the surface alginate.

Finally the alginate is reliquified by treating the droplet in a chelating agent to remove the calcium ions. Other methods use cells in a calcium solution to be dropped into a alginate solution, thus creating a hollow alginate sphere. A similar approach involves cells in a chitosan solution dropped into alginate, also creating hollow spheres.

Microencapsulated cells are easily propagated in stirred tank reactors and, with beads sizes in the range of 150-1500 μm in diameter, are easily retained in a perfused reactor using a fine-meshed screen. The ratio of capsule volume to total media volume can kept from as dense as 1:2 to 1:10. With intracapsular cell densities of up to 10^8, the effective cell density in the culture is $1\text{-}5 \times 10^7$.

The advantages of microencapsulation over other processes include the protection from the deleterious effects of shear stresses which occur from sparging and agitation, the ability to easily retain beads for the purpose of using perfused systems, scale up is relatively straightforward and the ability to use the beads for implantation.

6. Perfused attachment systems

Perfusion refers to continuous flow at a steady rate, through or over a population of cells (of a physiological nutrient solution). It implies the retention of the cells within the culture unit as opposed to continuous-flow culture which washes the cells out with the withdrawn media (*e.g.*, chemostat). The idea of perfusion has been known since the beginning of the century, and has been applied to keep small pieces of tissue viable for extended microscopic observation. The technique was initiated to mimic the cells milieu *in vivo* where cells are continuously supplied with blood, lymph, or other body fluids. Without perfusion, cells in culture go through alternating phases of being fed and starved, thus limiting full expression of their growth and metabolic potential. The current use of perfused culture is to grow cells at high densities (*i.e.*, $0.1\text{-}5 \times 10^8$ cells/ml). In order to increase densities beyond 2-4×10^6 cells/ml (or 2×10^5 cells/cm^2), the medium has to be constantly replaced with a fresh supply in order to make up for nutritional deficiencies and to remove toxic products. Perfusion allows for a far better control of the culture environment (pH, pO_2, nutrient levels, *etc.*) and is a means of significantly increasing the utilization of the surface area within a culture for cell attachment.

Microcarrier and microencapsulated cultures are readily adapted to perfused reactors but, as noted above, these culture methods lack the capacity to meet the demand for cell densities above 10^8 cells/ml. Such densities will provide for the advantage of high product titer in the medium (facilitating downstream processing), a smaller culture system (lowering facility needs), and a better medium utilization (yielding savings in serum and other expensive additives). Supporting cells at high density requires efficient perfusion techniques to prevent the development of non-homogeneity.

The cells of the present invention may, irrespective of the culture method chosen, be used in protein production and as cells for *in vitro* cellular assays and screens as part of drug development protocols.

B. *In vitro* Assays, Screens And Drug Development

Many cells produce biologically active molecules *in vivo* that are secreted and take effect at locations distal to the secretory cell. Neuroendocrine cells, by definition, have sorting mechanisms, whereby a given polypeptide or protein, destined for secretion, is targeted to the regulated secretory pathway or the default constitutive secretory pathway. Loss or impaired function of neuroendocrine cells is associated with a variety of human diseases and disorders.

In all the cases of secretory cell function disorder, there are significant drawbacks in the currently available treatment regimens, which generally rely on injections and suffers from the additional drawbacks of proper dosing, loss of drug efficacy, drug side effects, and patient compliance. There is a need for both new and effective pharmaceutical agents for the prevention and treatment of neuroendocrine based disease and in the effectiveness with which such agents prevent or retard the onset of complications. Certain uses of the immortal human neuroendocrine cells of the present invention are directed toward addressing these needs.

Immortal human neuroendocrine cell lines that reflect the *in vivo* production and secretion of proteins can be employed in the identification of modulators of regulated protein secretion. These aspects of the present invention are designed to take advantage of the secretory machinery of immortal human neuroendocrine cells for the purpose of screening for modulators of secretory function. A variety of different modifications may be made to these cells to make them more suitable candidates for drug screening. Additional aspects regarding the use of engineered human neuroendocrine cells in drug screening are found in U.S. Provisional Patent Applications Serial Nos. 60/072,556 and 60/087,848 (entitled "Recombinant Cell Lines For Drug Screening"; Attorney Docket Nos. BTGN:043PZ1 and BTGN:043PZ2), filed January 12, 1998 and June 3, 1998, respectively, and in U.S. Patent Application Serial No. 09/___,___ (entitled "Recombinant Cell Lines For Drug Screening"; Attorney Docket No. BTGN:055), filed January 11, 1999, the entire disclosures of which are incorporated herein by reference without disclaimer.

The development of therapeutic agents that target the pancreatic β–cell in patients with NIDDM has been hampered by an insufficiency of *in vitro* assays that are biologically relevant, stable, and allow for high through-put. From the perspective of fidelity to the target biology, the preferred material for *in vitro* screens for the purposes of identifying therapeutics is functional islets from human cadavers. However, such use of human islets is impractical due to extremely limited amounts of material, age-related difference in β–cell function, and complicated as well by the batch-to batch variation that has been observed for islet preparations (Jansson *et al.*, 1995; McClenaghan *et al.*, 1996).

One material that could be potentially used in biological screens to identify candidate agents as therapies for NIDDM are rodent β–cell lines. Several such cell lines have been established, maintained for extended periods *in vitro*, and characterized with regard to the synthesis and regulated secretion of insulin. The cell lines include those established from the pancreatic islets of rat (Chick *et al.*, 1977; Gazdar *et al.*, 1980; McClenaghan *et al.*, 1996) hamster (Ashcroft *et al.*, 1986), and mouse (Miyazaki *et al.*, 1990; Knaack *et al.*, 1994). These cell lines and their clonal derivatives potentially provide an unlimited supply of material; however, they are deficient in other properties important for their use as screening tools for therapeutic agents for diabetes.

All β–cell lines described to date are deficient in key functional aspects when compared to islet β–cells. Insulin content is decreased and proinsulin processing is often impaired (Poitout *et al.,* 1996). In β–cell lines the threshold or magnitude of glucose-stimulated insulin secretion (GSIS) and response to agents that can potentiate GSIS are often not representative of primary β–cells (Newgard, 1996). In addition, many β–cell lines express peptides that are not expressed by the primary β–cell such as somatostatin, pancreatic polypeptide, and glucagon (Madsen *et al.,* 1986).

One of the most ubiquitous properties of established β–cell lines is phenotypic instability such that, with extended cultivation, the cells lose those traits that are singularly β–cell in nature. HIT cells, many βTC cell lines, and unengineered RIN cell lines have less insulin content than a normal β–cell (Radvanyi *et al.,* 1993), and this level has been reported to drop if the cells are maintained in culture for multiple population doublings (Clark *et al.,* 1990; Poitout *et al.,* 1996). One study reports that HIT cells lose responsiveness to glucose, arginine, and various secretagogues with serial passages (Zhang *et al.,* 1989). Many βTC cell lines display relatively normal GSIS at early passages, but with continuous propagation these cells acquire aberrant insulin secretion that is characterized by a hypersensitivity to glucose (Efrat *et al.,* 1988; Efrat *et al.,* 1993; Poitout *et al.,* 1996).

The present invention, however, provides immortal human neuroendocrine cells lines with a phenotypic integrity that allows them to be used as screening tools for the identification of novel substances that can be employed in the modulation of secretory function that is manifest in a number of diseased states including diabetes. Further, these cells may be engineered to ensure that glucose sensing and responsiveness is maintained over a period of time, *i.e.*, indefinitely. The components for such a system, and methods of making and using such cell lines are set forth in detail below.

1. The use of engineered β–cell lines for drug discovery in NIDDM

The onset and progression of NIDDM are marked by discreet stages of β–cell dysfunction and failure with defects in insulin secretion presenting throughout the disease (Porte 1991; Granner and O'Brien 1992; Polonsky 1995; Polonsky *et al.*, 1996). The early phases of NIDDM often are characterized by insulin resistance and a compensatory increase in the secretion of insulin. As hyperinsulinemia fails to completely overcome insulin resistance, mild fasting hyperglycemia and impaired glucose tolerance become detectable. With progression of the diabetic state, hypoinsulinemia presents. The first-phase of insulin secretion is short in duration and facilitates immediate glucose disposal. Its loss, typically early in NIDDM, results in postprandial hyperglycemia. Eventually, the second phase of glucose-stimulated insulin secretion (GSIS) becomes impaired and results in overt NIDDM with fasting hyperglycemia. Other defects in insulin secretion that are often present in NIDDM are abnormal, pulsatile insulin secretion, an increase in the plasma of ratio of proinsulin to insulin, and impairments of various secretagogues to potentiate GSIS (Porte 1991; Granner and O'Brien 1992; Polonsky 1995; Polonsky *et al.*, 1996).

It is postulated that the ongoing decline of the β–cell in NIDDM is related to perturbations in glucose homeostasis and fatty acid metabolism (Porte 1991), or as has more recently been termed "glucolipotoxicity" (DeFronzo, 1997). Glucolipotoxicity refers to the detrimental effects that sustained elevations of plasma glucose and free fatty acids have on β–cell metabolism. The resulting disruption of β–cell fuel metabolism leads to faulty insulin secretion and contributes to ongoing β–cell failure.

Optimal glucose-sensing and subsequent insulin secretion have been linked to two key β–cell proteins: the glucose transporter, type 2 (GLUT-2) and hexokinase, type IV, (glucokinase). The relatively low affinity of these proteins for glucose allows them to be rate-limiting in the transport and metabolism of glucose and provides the glucose-sensing required for physiologically relevant insulin secretion (Newgard and McGarry 1995; Matschinsky 1996). Cytosolic long chain fatty acyl-CoA esters and free fatty acids are potentiators of GSIS (DeFronzo, 1997). Partitioning of fatty acyl-CoA molecules between the cytoplasm and mitochondria is regulated by glucose. Basal glucose levels stimulate transport into the mitochondria and β-oxidation, and elevations of intracellular glucose promote increases in cytoplasmic concentrations of long-chain fatty acyl CoA esters and potentiation of GSIS.

A key point for regulating the partitioning of fatty acids is carnitine palmitoyl transferase l (CPT-1), the protein that transports fatty acyl-CoA molecules into the mitochondria from the cytoplasm. CPT-1 is inhibited by malonyl CoA, a metabolite that increases with increases in glucose metabolism (DeFronzo, 1997). Thus, the metabolism of glucose in the pancreatic β–cell is tightly coupled to the metabolism of fatty acids, both in normal physiology and in pathogenic states such as diabetes. Whereas short-term elevations in glucose and fatty acids are part of the normal signaling required for fuel homeostasis, chronic exposures are detrimental to β–cell physiology and could contribute to an inhibition of insulin secretion.

Fatty acid metabolism in the β–cell also seems to be regulated by the effects of leptin (Shimabukuro *et al.,* 1997a; Zhou *et al.,* 1997). Leptin is a peptide hormone synthesized in and secreted from adipocytes. It has dramatic effects on body composition by regulating food intake and thermogenesis. Rodent strains with defects either in leptin production or leptin receptors are obese and a have high incidence of NIDDM. Increases in plasma leptin levels have been shown to reduce body fat in several obese and non-obese rodent models. Leptin receptors are expressed in tissues throughout the body and have been shown to be present in pancreatic islets. Leptin has been shown to induce enzymes of fatty acid oxidation and deplete triglyceride pools in pancreatic islets (Shimabukuro *et al.,* 1997a; Zhou *et al.,* 1997). It is postulated that leptin functions in normal physiology to protect the β–cell from lipotoxicity and helps to prevent adipogenic diabetes (Shimabukuro *et al.,* 1997a, b; Zhou *et al.,* 1997). It is currently unknown if defects in leptin-regulated fatty acid metabolism are causally linked to progressive β–cell failure that is characteristic of NIDDM in humans.

Clearly, β–cell failure characteristic of NIDDM is a complex and multi-faceted process. Currently, the only pharmaceutical agents that target the β–cell in NIDDM are the sulfonylurea agents. These drugs treat the symptom of hyperglycemia by interacting with K^+-ATP channels to stimulate insulin secretion. However, long-term use of the sulfonylurea drugs often results in a loss of drug efficacy. No drugs are available for the following defects that are often seen in diabetes: correcting defects in fatty acid metabolism, preventing oxidative damage to β–cells, restoring the potency of glucose and other secretagogues in insulin secretion, stimulating insulin secretion via receptors other than SUR/KIR or internal signaling machinery, and preventing or retarding progressive β–cell failure.

Engineered immortal human neuroendocrine cell lines with stable phenotypes can be used to screen compounds that alter insulin secretion. These engineered cells afford the capacity of *in vivo* testing of candidate compounds for safety and efficacy.

2. Desired Properties of an Islet β–cell

Thus, in particular embodiments, the present invention provides an immortal human neuroendocrine secretory cell that may be employed in the identification of modulators of secretory function. Such compounds will be especially useful in a variety of diseased states in which secretory function has been impaired. An example of such a state would be the loss of β–cell integrity that is manifest in diabetes. Thus, immortalized secretory cells that have a stable neuroendocrine phenotype are used to identify compounds that will be useful in the regulating this secretory function.

There are a number of properties that are desired of a cell line that will be representative of a human islet β–cell. First, it is important to ensure that engineered cell lines have a measurable activity that accurately reflects the regulation and capacity of the secretory pathway. One way to achieve this endpoint is to express a transgenic polypeptide at high levels that is processed and secreted through the regulated secretory pathway. Such a peptide, by definition, will be detectable in the media of the cultured cells, and its secretion will be dependent of the stimulatory or inhibitory signals that are received and processed by the cell.

The regulated pathway of the β–cell encompasses both acute regulation of insulin secretion (*i.e.* a large increment between the unstimulated and stimulated states) and the complete processing of proinsulin to the mature insulin polypeptide. In islet β–cells, secretory granules allow the storage of insulin as a depot at the plasma membrane that can be released within seconds of arrival of a fuel-derived or hormonal signal, and also serve as the site of conversion of proinsulin to insulin by virtue of their high concentrations of the relevant convertases PC1 (also known as PC3) and PC2. The presence of secretory granules and retention of proinsulin processing capacity represent a major advantage of insulinoma and other neuroendocrine cell lines relative to cells less specialized for secretion of peptide hormones such as hepatoma cells or fibroblasts.

A second, and certainly central parameter, is for the cell to be equipped with a capacity for secretagogue sensing and responsiveness. For example, glucose responsiveness has several components that must be considered, including the appropriate threshold for the response (islet β–cells typically respond to glucose at concentrations in excess of the fasting level of 4-5 mM), rapid response dynamics (β-cells secrete insulin in response to glucose within minutes of its application and turn off insulin secretion nearly as rapidly when glucose is removed) and an appropriate magnitude of response.

Finally, it is imperative that engineered cell lines retain phenotypic and genotypic stability. This includes both genes that are inserted or deleted during the course of engineering and key endogenous genes. This is a significant limitation on current technology.

3. Screening For Modulators Of Secretion

The immortalized human neuroendocrine secretory cell lines described by the present invention have a stable neuroendocrine phenotype. They are able to providing a measurable secretion of the secretory product and secretagogue responsive, thereby indicating that these cell lines respond to modulators of secretory function. Therefore, within certain embodiments of the invention, methods are provided for screening for modulators of secretory function. Such methods may use the cells of the present invention either as adherent cells on a culture dish, as part of an alginate biomatrix, in suspension culture or in any other form that permits the secretion of the polypeptide to be monitored. These cells are then used as reagents to screen small molecule and peptide libraries to identify modulators of secretory function.

Secretory function embodies all aspects of the cell's capacity to sense the extracellular milieu, respond to that milieu via the activation and inhibition of a variety of intracellular signaling mechanisms, and accordingly regulate the secretion of a peptide or hormone from the secretory pathway. Regulation from the secretory pathway can occur at any phase in the synthesis and release of a peptide or hormone including gene transcription; stability of the mRNA; translation; post-translational modifications such as proteolytic processing, formation of disulfide bonds, amidation, and glycosylation; trafficking through the secretory tubules and vesicles; storage within the secretory granule; membrane fusions, and exocytosis. In particular embodiments, the secretory function may be manifest as the secretion of a particular polypeptide from a secretory cell.

The polypeptide is generally secreted into the media of the cells, from where it can be quantified using any of a number of techniques. The polypeptide may be detected directly from the media using for example, ELISA, RIA and the like. Alternatively, the polypeptide may be purified prior to detection according to known methods, such as precipitation (*e.g.*, ammonium sulfate), HPLC, immunoprecipitation, ion exchange chromatography, affinity chromatography (including immunoaffinity chromatography) or various size separations (sedimentation, gel electrophoresis, gel filtration). Such techniques of polypeptide separation are well known to those of skill in the art. The purified polypeptide may then quantified through immunodetection methods, biological activity, or radioisotope labeling. These techniques are described herein below.

a. Assay Formats

Stimulators of Secretory Function

The present invention provides methods of screening for stimulators of secretory function, by monitoring secretory function in the absence of the candidate substance and comparing such results to the assay performed in the presence of candidate secretory function stimulators.

In certain embodiments, the present invention concerns a method for identifying such stimulators. It is contemplated that this screening technique will prove useful in the general identification of a compound that will serve the purpose of promoting, augmenting or increasing the secretion of, for example, a polypeptide from a secretory cell as exemplified by the immortalized human neuroendocrine secretory cells of the present invention. Such compounds will be useful in the treatment of various secretory disorders resulting from impaired secretory function, such as for example, diabetes, Parkinson's disease, athyrotic cretinism and Adison's disease.

In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to stimulate the secretory function of immortalized cells that either naturally secrete molecules or have been engineered to possess secretory function as described herein. The method including generally the steps of:

(a) providing at least one immortalized cell having stable secretory function;
(b) contacting said cell with said candidate substance;
(c) measuring the secretory function of said cell; and
(d) comparing the secretory function of the cell in step (c) with the secretory function of the cell of step (a).

To identify a candidate substance as being capable of stimulating secretory function in the assay above, one would measure or determine the secretory function in the absence of the added candidate substance by determining the secretion of the desired molecule. One would then add the candidate substance to the cell and determine the secretory function in the presence of the candidate substance. A candidate substance which increases the secretory function or capacity relative to the secretory function in its absence, is indicative of a candidate substance with stimulatory capability.

Secretory function may be determined by measuring the amount of secreted molecule. In particular embodiments, the secreted molecule will be a polypeptide such as an amidated polypeptide, glycosylated polypeptide, a hormone or a growth factor. In such circumstances these molecules may be detected using any of a number of techniques well known to those of skill in the art as described herein below. Secretory function may also be monitored indirectly by measuring, for example, intracellular calcium or membrane potential with the use of fluorescent indicator molecules.

Inhibitors of Secretory Function

These assays may be set up in much the same manner as those described above in assays for secretory function stimulators. In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to have an inhibitory or even antagonistic effect on secretion from the immortalized cells described herein. The method including generally the steps of:

(a) providing at least one immortalized cell having secretory function;
(b) contacting said cell with said candidate substance;
(c) measuring the secretory function of said cell; and
(d) comparing the secretory function of the cell in step (c) with the secretory function of the cell of step (a).

To identify a candidate substance as being capable of inhibiting secretory function one would measure or determine such a secretory activity in the absence of the added candidate substance and monitoring the secretory function. One would then add the candidate inhibitory substance to the cell and determine the secretory function in the presence of the candidate inhibitory substance. A candidate substance which is inhibitory would decrease the secretion from said cell, relative to the amount of secretion in its absence.

Candidate Substances

As used herein the term "candidate substance" refers to any molecule that is capable of modulating secretory function. The candidate substance may be a protein or fragment thereof, a small molecule inhibitor, or even a nucleic acid molecule. It may prove to be the case that the most useful pharmacological compounds for identification through application of the screening assay will be compounds that are structurally related to other known modulators of secretion. The active compounds may include fragments or parts of naturally-occurring compounds or may be only found as active combinations of known compounds which are otherwise inactive. However, prior to testing of such compounds in humans or animal models, it will be necessary to test a variety of candidates to determine which have potential.

Accordingly, the active compounds may include fragments or parts of naturally-occurring compounds or may be found as active combinations of known compounds which are otherwise inactive. Accordingly, the present invention provides screening assays to identify agents which stimulate or inhibit cellular secretion, it is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be polypeptide, polynucleotide, small molecule inhibitors or any other compounds that may be designed through rational drug design starting from known secretagogues or inhibitors of secretory function.

The candidate screening assays are relatively simple to set up and perform, with the provision of the immortal human neuroendocrine cells of the present invention and in light of the present disclosure. Thus, in assaying for a candidate substance, after obtaining an immortalized secretory cell of the present invention, one will admix a candidate substance with the cell, under conditions which would allow measurable secretion to occur. In this fashion, one can measure the ability of the candidate substance to stimulate the secretory function of the cell in the absence of the candidate substance. Likewise, in assays for inhibitors after obtaining an immortalized secretory cell, the candidate substance is admixed with the cell. In this fashion the ability of the candidate inhibitory substance to reduce, abolish, or otherwise diminish secretion from said cell may be detected.

"Effective amounts" in certain circumstances are those amounts effective to reproducibly stimulate secretion from the cell in comparison to their normal levels. Compounds that achieve significant appropriate changes in activity will be used.

Significant increase in secretory function, *e.g.*, as measured using RIA, HPLC, ELISA, biological activity and the like are represented by an increase/decrease in secretion of at least about 30%-40%, and most preferably, by increases of at least about 50%, with higher values of course being possible. The active compounds of the present invention also may be used for the generation of antibodies which may then be used in analytical and preparatory techniques for detecting and quantifying further such inhibitors.

It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

b. Purification of Secreted Products

Purification techniques are well known to those of skill in the art, and will be used to purify the molecules secreted from the immortalized secretory cells of the present invention. These techniques tend to involve the separation of the secreted protein or other secretory molecule from other components of the mixture. Having separated the secreted product from the other components, the sample may be purified using chromatographic and electrophoretic techniques to achieve complete purification. Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

In certain aspects, the secreted molecule is a polypeptide and may be isolated from the conditioned media and analyzing the extracts by HPLC as described (Halban, *et al.,* 1986, Sizonenko and Halban, 1991). Solvent systems, gradients and flow rates used were as described by Halban, *et al.,* (1986) however it is well within the skill of the ordinary person in the art to adapt the chromatography conditions to suit individual need. Standards may be used to obtain optimization of chromatography conditions and methods.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of a secreted product. The term "purified" as used herein, is intended to refer to a composition, isolatable from other components, wherein the product is purified to any degree relative to its naturally-obtainable state, *i.e.*, in this case, relative to its purity within a hepatocyte or β–cell extract. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the number of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number". The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater -fold purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.,* 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Concanavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed briefly below, and is within the skill of the ordinary artisan in light of the present disclosure.

c. Methods of Detection

The present invention encompasses methods for determining the effects of active compounds on the secretory function of the immortalized secretory cells of the present invention. Generally, this will be achieved by determining the secretion of, for example a secretory polypeptide, of the immortalized secretory cell in the presence of the active compounds and comparing the level of secretion with those levels observed in normal cells of the same type. In this manner the secretory function of the immortalized secretory cells may be quantitated.

The immunodetection methods of the present invention have evident utility in the detection of polypeptide secretion. Here, a sample containing the secreted moiety is contacted with a corresponding antibody. Thus, in an exemplary assay, a modulator screening assay is performed in which cells secreting a polypeptide are exposed to a test or candidate substance under suitable conditions and for a time sufficient to permit the agent to effect secretion of the polypeptide. The secretion of the polypeptide is then detected by incubating the reaction mixture with for example a specific antibody, which antibody may be labeled directly or may be detected secondarily, (*e.g.*, using a labeled idiotypic or species specific antibody) under conditions that permit the formation of immune complexes between the polypeptide and its specific antibody. The test reaction is compared to a control reaction which lacks the test sample. To complete the modulator screening assay, the presence and/or amount of complexes formed between the polypeptide and the antibody is detected in the test sample (*e.g.* by determining the presence or amount of label bound directly to the antibody or to a secondary antibody directed against the primary antibody). Within this exemplary assay, agents that inhibit polypeptide secretion will demonstrate a reduced binding with polypeptide-specific antibodies relative to the control sample and agents that induce or stimulate polypeptide secretion will demonstrate an increased binding with specific antibodies relative to the control sample.

Those of skill in the art are very familiar with differentiating between significant secretion of a protein, which represents a positive identification, and low level or background secretion of such a protein.

Immunodetection Methods

In still further embodiments, the present invention concerns immunodetection methods for binding, purifying, removing, quantifying or otherwise generally detecting biologically the secreted components. The steps of various useful immunodetection methods have been described in the scientific literature and are well known to those of skill in the art.

In general, the immunobinding methods include obtaining a sample suspected of containing a compound of interest (i.e. the secreted molecule), and contacting the sample with an antibody under conditions effective to allow the formation of immunocomplexes. The immunobinding methods include methods for detecting or quantifying the amount of a reactive component in a sample, which methods require the detection or quantitation of any immune complexes formed during the binding process. Here, one would obtain a sample to be measured as containing a secreted molecule, and contact the sample with an antibody or encoded protein or peptide, as the case may be, and then detect or quantify the amount of immune complexes formed under the specific conditions.

In terms of detection, the biological sample analyzed may be any sample that is suspected of containing a secreted molecule from the immortalized, human neuroendocrine secretory cells of the present invention. Such a cell may be an immortalized human neuroendocrine cell, an immortalized human pancreatic β–cell, or even any biological fluid that comes into contact with the secretory cells *in vivo*.

Contacting the chosen sample with the antibody under conditions effective and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the composition to the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, *i.e.*, to bind to, any antigens present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any radioactive, fluorescent, biological or enzymatic tags or labels of standard use in the art. U.S. Patents concerning the use of such labels include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

The secreted protein, peptide or corresponding antibody employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined.

Alternatively, the first added component that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the encoded protein, peptide or corresponding antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the encoded protein, peptide or corresponding antibody is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under conditions effective and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if desired.

ELISA

It is contemplated that the secreted proteins or peptides of the invention will be detected in a preferred embodiment in ELISA assays. Antibodies against such secreted proteins are readily available to those of skill in the art. Immunoassays, in their most simple and direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) known in the art.

In one exemplary ELISA, antibodies binding to the secreted proteins of the invention are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition containing the secreted polypeptide is added to the wells. After binding and washing to remove non-specifically bound immune complexes, the bound antibody may be detected. Detection is generally achieved by the addition of a second antibody specific for the target protein, that is linked to a detectable label. This type of ELISA is a "sandwich ELISA". Detection may also be achieved by the addition of a second antibody, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

In another exemplary ELISA, the samples containing the secreted polypeptide are immobilized onto the well surface and then contacted with the antibodies of the invention. After binding and washing to remove non-specifically bound immune complexes, the bound antigen is detected. Where the initial antibodies are linked to a detectable label, the immune complexes may be detected directly. Again, the immune complexes may be detected using a second antibody that has binding affinity for the first antibody, with the second antibody being linked to a detectable label.

Another ELISA in which the proteins or peptides are immobilized, involves the use of antibody competition in the detection. In this ELISA, labeled antibodies are added to the wells, allowed to bind to the secreted protein, and detected by means of their label. The amount of marker antigen in an unknown sample is then determined by mixing the sample with the labeled antibodies before or during incubation with coated wells. The presence of marker antigen in the sample acts to reduce the amount of antibody available for binding to the well and thus reduces the ultimate signal. This is appropriate for detecting antibodies in an unknown sample, where the unlabeled antibodies bind to the antigen-coated wells and also reduces the amount of antigen available to bind the labeled antibodies.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. These are described as follows:

In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of milk powder. The coating of nonspecific adsorption sites on the immobilizing surface reduces the background caused by nonspecific binding of antisera to the surface.

In ELISAs, it is more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of a protein or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the control and/or clinical or biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween™. These added agents also tend to assist in the reduction of nonspecific background.

The "suitable" conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25° to 27°C, or may be overnight at about 4°C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween™, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this label will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immune complex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immune complex formation (*e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween™).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azido-di-(3-ethyl-benzthiazoline-6-sulfonic acid [ABTS] and H_2O_2, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra spectrophotometer.

In a one embodiment, the screening assay uses live cells in the 96-well format has long been the standard used as the format lends itself to automation and robotics handling. The 96-well plate format allows for a variety of different candidate substances to be tested in one plate. However, use of live cells for the purpose of drug screening has inherent problems associated with the handling of the cells. Handling attachment dependent cell culture in the 96-well format becomes difficult when there is a need for several exchanges of solution. The forces of surface tension associated with the meniscus on the well wall stress and even damages cells on the bottom of the well as aqueous solutions are removed or added. The shear forces created by a suction device (*e.g.*, a pipet tip) as it is close to the cell layer removing the last microliter of solution may also damage and remove cells. In order to overcome these problems, the cells are encapsulated in highly porous, biocompatible hydrogels in a bead form. In a preferred assay format, the encapsulated cells are placed in a 96-well plates that incorporates a filter-bottom. The cells are then incubated with the candidate substance for a suitable period of time to allow the cell to secrete the polypeptide of interest. This incubation step is followed by the harvesting of the media from the cells by the application of a vacuum below the plate to empty all wells in one step. The collected samples are then assayed for the presence of the secreted peptide using standard ELISA techniques well known to those of skill in the art.

In other preferred embodiments, the detection assays may be radioimmunoassays as described by various groups (Halban *et al.*, 1986; Pieber, *et al.*, 1994). Standard commercially available radioimmunoassays are available from Coat-a-count, Diagnostic Products Corp., Los Angeles for insulin, and rat amylin immunoassay (Peninsula Laboratories, EIAH-7323.

Immunoreactive species of glucagon, glucagon-like peptide 1 (7-37, non amidated) and glucagon-like peptide (7-36, amide) were determined as described by the suppliers of the respective commercial kits (all purchased from Peninsula Laboratories Inc. Cat #s RIK-7165, RIK-7123 and RIK-7168, respectively)

Non-Immunologic Methods

Alternatively, one may employ non-immunologic procedures in the measuring the secretory function according to the of the present invention. For example, when examining molecules that are involved in receptor interactions, it is possible to set up assays that look at the occupancy of relevant receptor molecules. This can be performed, for example, by using labeled ligand molecules that will be compete with the ligand (stimulators and inhibitor) in the sample. The more ligand in the sample, the less labeled receptor that will be bound to the receptor. Such studies can be performed on whole cells as well as on purified receptors. Labels include radiolabels, fluorescent labels and chemiluminescent labels.

Other non-immunologic forms of diagnostic assays include those that look for the presence of biological activity of the secreted polypeptide.

XIII. *In vivo* Uses

A. Pharmaceutically Acceptable Formulations

Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions of the stable cells in a form appropriate for the intended application, which will most usually be within a selectively permeable membrane. Nonetheless, the cells will generally be prepared as a composition that is essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render stable cells suitable for introduction into a patient or for animal testing within their selectively permeable membrane, implantable device or other delivery vehicle. Aqueous compositions of the present invention comprise an effective amount of stable neuroendocrine cells dispersed in a pharmaceutically acceptable carrier or aqueous medium, and preferably encapsulated.

The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. As used herein, this term is particularly intended to include biocompatible implantable devices and encapsulated cell populations. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Under ordinary conditions of storage and use, the cell preparations may further contain a preservative to prevent growth of microorganisms. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components in the pharmaceutical are adjusted according to well-known parameters.

B. Cell-Based Delivery and Devices

The engineered cells of the present invention may be introduced into animals, including human subjects, with certain needs, such as patients with insulin-dependent diabetes. In the diabetic treatment aspects, ideally cells are engineered to achieve glucose dose responsiveness resembling that of islets. However, other cells will also achieve advantages in accordance with the invention. It should be pointed out that the studies of Madsen and coworkers have shown that implantation of poorly differentiated rat insulinoma cells into animals results in a return to a more differentiated state, marked by enhanced insulin secretion in response to metabolic fuels (Madsen *et al.*, 1988). These studies suggest that exposure of engineered cell lines to the *in vivo* milieu may have some effects on their response(s) to secretagogues.

A preferred method of administration involves the encapsulation of the engineered cells in a biocompatible coating. In this approach, the cells are entrapped in a capsular coating that protects the contents from immunological responses. One preferred encapsulation technique involves encapsulation with alginate-polylysine-alginate. Capsules made employing this technique generally have a diameter of approximately 1 mm and should contain several hundred cells.

Cells may thus be implanted using the alginate-polylysine encapsulation technique of O'Shea and Sun (1986), with modifications, as later described by Fritschy *et al.* (1991; both references incorporated herein by reference). The engineered cells are suspended in 1.3% sodium alginate and encapsulated by extrusion of drops of the cell/alginate suspension through a syringe into $CaCl_2$. After several washing steps, the droplets are suspended in polylysine and re-washed. The alginate within the capsules is then reliquified by suspension in 1 mM EGTA and then rewashed with Krebs balanced salt buffer.

An alternative approach is to seed Amicon fibers with stable cells of the present invention. The cells become enmeshed in the fibers, which are semipermeable, and are thus protected in a manner similar to the micro encapsulates (Altman *et al.*, 1986; incorporated herein by reference). After successful encapsulation or fiber seeding, the cells may be implanted intraperitoneally, usually by injection into the peritoneal cavity through a large gauge needle (23 gauge).

A variety of other encapsulation technologies have been developed that are applicable to the practice of the present invention (see, *e.g.*, Lacy *et al.*, 1991; Sullivan *et al.*, 1991; WO 91/10470; WO 91/10425; WO 90/15637; WO 90/02580; U.S. Patent 5,011,472; U.S. Patent 4,892,538; and WO 89/01967; each of the foregoing being incorporated by reference).

Lacy *et. al.* (1991) encapsulated rat islets in hollow acrylic fibers and immobilized these in alginate hydrogel. Following intraperitoneal transplantation of the encapsulated islets into diabetic mice, normoglycemia was reportedly restored. Similar results were also obtained using subcutaneous implants that had an appropriately constructed outer surface on the fibers. It is therefore contemplated that engineered cells of the present invention may also be straightforwardly "transplanted" into a mammal by similar subcutaneous injection.

Sullivan *et. al.* (1991) reported the development of a biohybrid perfused "artificial pancreas", which encapsulates islet tissue in a selectively permeable membrane. In these studies, a tubular semi-permeable membrane was coiled inside a protective housing to provide a compartment for the islet cells. Each end of the membrane was then connected to an arterial polytetrafluoroethylene (PTFE) graft that extended beyond the housing and joined the device to the vascular system as an arteriovenous shunt. The implantation of such a device containing islet allografts into pancreatectomized dogs was reported to result in the control of fasting glucose levels in 6/10 animals. Grafts of this type encapsulating engineered cells could also be used in accordance with the present invention.

The company Cytotherapeutics has developed encapsulation technologies that are now commercially available that are envisioned for use in the application of the present invention. A vascular device has also been developed by Biohybrid, of Shrewsbury, Mass., that can be used with the technology of the present invention. Other implantable containment apparati contemplated for use with in the application of the present invention are described in U.S. Patent Nos. 5,626,561, 5,787,900 and 5,843,069, each of which are incorporated herein by reference.

Implantation employing such encapsulation techniques are preferred for a variety of reasons. For example, transplantation of islets into animal models of diabetes by this method has been shown to significantly increase the period of normal glycemic control, by prolonging xenograft survival compared to unencapsulated islets (O'Shea and Sun, 1986; Fritschy *et al.*, 1991). Also, encapsulation will prevent uncontrolled proliferation of clonal cells. Capsules containing cells are implanted (approximately 1,000-10,000/animal) intraperitoneally and blood samples taken daily for monitoring of blood glucose and insulin.

An alternate approach to encapsulation is to simply inject glucose-sensing cells into the scapular region or peritoneal cavity of diabetic mice or rats, where these cells are reported to form tumors (Sato *et al.*, 1962). Implantation by this approach may circumvent problems with viability or function, at least for the short term, that may be encountered with the encapsulation strategy. This approach will allow testing of the function of the cells in experimental animals, which is a viable use of the present invention, but certainly is not applicable as an ultimate strategy for treating human diabetes. Nonetheless, as a pre-clinical test, this will be understood to have significant utility.

In summary, biohybrid artificial organs encompass all devices which substitute for an organ or tissue function and incorporate both synthetic materials and living cells. Implantable immunoisolation devices will preferably be used in forms in which the tissue is protected from immune rejection by enclosure within a semipermeable membrane. Those of skill in the art will understand device design and performance, as it relates to maintenance of cell viability and function. Attention is to be focused on oxygen supply, tissue density and the development of materials that induce neovascularization at the host tissue-membrane interface; and also on protection from immune rejection. Membrane properties may even be further adapted to prevent immune rejection, thus creating clinically useful implantable immunoisolation devices.

C. Treatment

An effective amount of the stable cells is determined based on the intended goal. The term "unit dose" refers to a physically discrete unit suitable for use in a subject, each unit containing a predetermined quantity of the therapeutic composition calculated to produce the desired response in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the subject to be treated, the state of the subject, and the protection desired. Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual.

D. Glycemic Control

Insulin-dependent diabetes mellitus (IDDM) is a multifactorial complex disorder that is caused by autoimmune destruction of pancreatic islet β-cells. Not only does this autoimmune attack result in the loss of insulin stores, it also eliminates the tight regulation of insulin delivery afforded by the β-cells, cells that are designed to recognize and integrate a complex array of metabolic and hormonal signals for maintenance of fuel homeostasis.

Glucose is the predominant fuel source for the central nervous system (CNS); yet tissues of the CNS are incapable of synthesizing glucose and can store the metabolic fuel for only minutes. The concentration of glucose in the CNS is maintained by the tight regulation of plasma glucose levels which are kept within a narrow range (70-150 mg/dL or 3.9 - 8.3 mMol/L) by the interplay among multiple molecules and signaling pathways within the glucoregulatory system.

Glucoregulatory failure caused by insulin deficiency and resulting in hyperglycemia is the common affliction of diabetes mellitus. By contrast, hypoglycemia is a relatively rare clinical disorder except when it occurs as a side effect of diabetes treatment. Thus, in order to find an effective treatment for diabetes, the hypoglycemic effects of insulin and sulphonylureas cannot be overlooked and need to be remedied.

Virtually all patients with IDDM experience deficient glucagon-secretory responses to hypoglycemia, and have impaired epinephrine-secretory response to decreases in plasma glucose. Thus, the high incidence of hypoglycemia in these patients is a consequence of multiple defects in fuel homeostasis resulting from imperfect insulin replacement and failures to compensate for low plasma glucose. The restoration of glucose counter-regulatory responses in IDDM patients could provide a means to reduce hypoglycemic episodes and thereby afford a safer pursuit of tight glycemic control. One preferred embodiment for such a goal could be achieved by cell-based delivery of glucagon. α-cells could be engineered to express human glucagon, and made responsive to epinephrine by the expression of α1- or β-adrenergic receptors or vasopressin receptors. In a second embodiment, β-cells could be engineered in such a manner as to decrease the secretion of insulin in response to hypoglycemia. In such an embodiments, the β-cells are generally engineered to express receptors for agents that will suppress the secretion of insulin, for example glucocorticoid receptors, pancreatic polypeptide receptors and α2-adrenergic receptors.

Thus, in a broad sense, certain aspects of the present invention are directed towards diabetes therapy that will balance the hyperglycemic effects of loss of β-cell function and the hypoglycemic effects of insulin therapies, thereby normalizing plasma glucose. Additional aspects of glycemic control contemplated for use in the present invention are found in U.S. Provisional Patent Applications Serial Nos. 60/071,193 and 60/087,821 (entitled "Compositions and Methods for Glycemic Control Of Cells"; Attorney Docket Nos. BTGN:042PZ1 and BTGN:042PZ2), filed January 12, 1998 and June 3, 1998, respectively, and U.S. Patent Application Serial No. 09/___,___ (entitled "Compositions and Methods for Regulated Secretion From Neuroendocrine Cell Lines"; Attorncy Docket No. BTGN:054), filed January 11, 1999, the entire disclosures of which are incorporated herein by reference without disclaimer.

1. The Factors involved in Glucose Regulation

The regulatory mechanisms that maintain systemic glucose balance involve hormonal, neural and substrate factors. Just as insulin secretion from the β-cell is complexly regulated, there are multiple hormones and peptides (in addition to insulin) that are involved in the regulation of plasma glucose concentrations including glucagon, epinephrine, cortisol and pancreatic polypeptide. Many of these molecules mediate their effects by direct effects on the pancreatic β-cell, while others mediate their effects at the levels of hepatic glucose metabolism, dietary glucose absorption, and indirect endocrine effects. The interplay between these signals in maintaining normal glucose balances is discussed in further detail herein below.

Glucoregulatory hormones include insulin, glucagon, epinephrine, and cortisol. Insulin is the major hormone responsible for lowering plasma glucose concentrations (Service, 1983: Marks and Rose, 1981; Cryer, 1988; Cryer *et al.*, 1989). It inhibits glucose synthesis and stimulates glucose utilization (Rizza *et al.*, 1981). Pancreatic β-cells secrete insulin into the hepatic portal circulatory system for effects on the liver and at various peripheral organs. Overall, insulin inhibits gluconeogenesis and glycogenolysis at the liver and converts the liver into an organ of glucose uptake.

Glucose raising or "glucose counter-regulatory hormones" include glucagon, epinephrine, norepinephrine, pancreatic polypeptide, vasopressin and cortisol.

Glucagon is secreted by the α-cells of the pancreatic islets into the hepatic circulation, its physiological site of action being the liver (Service, 1983: Marks and Rose, 1981; Cryer, 1988; Cryer *et al.*, 1989). It potently activates gluconeogenesis and glycogenolysis and mediates a transient increase of hepatic glucose production within minutes. Even in the event of sustained glucagon release, hepatic glucose production returns to basal rates in approximately 90 minutes although the hormone continues to support glucose production (Rizza and Gerich, 1979). Glucagon-induce hyperglycemia is transient because the glucagon-induced increase in glycogenolysis does not persists, an effect likely to be due to the glucose induced insulin secretion coupled with the autoregulatory effects of hyperglycemia (Gherrington *et al.*, 1981).

Epinephrine is an adrenomedullary hormone that also mediates a hyperglycemic effect. It has a dual role in that it stimulates hepatic glucose production and limits glucose utilization. The direct and indirect actions of epinephrine are mediated through the α- and β-adrenergic mechanisms in humans (Rizza *et al.*, 1980; Berk *et al.*, 1985; Clutter *et al.*, 1988). The α-adrenergic limitation of insulin secretion is an action of epinephrine. It allows the hyperglycemic response to occur, although an increase in insulin secretion in response to increased levels of plasma glucose limits the magnitude of hyperglycemia (Berk *et al.*, 1985; Clutter *et al.*, 1988). The β-adrenergic stimulation of glucagon secretion by epinephrine also occurs (Gerich *et al.*, 1976; Gray *et al.*, 1980) but its contribution to hyperglycemic effects of epinephrine are relatively minor (Berk *et al.*, 1985; Clutter *et al.*, 1988). Further, epinephrine also acts directly to increase hepatic gluconeogenesis and glycogenolysis. This direct hepatic effect is mediated through β-adrenergic mechanisms (Rizza *et al.*, 1980; Clutter *et al.*, 1988; Deibert and DeFronzo, 1980) although direct α-adrenergic stimulation of hepatic glucose has also been reported (Rosen *et al.*, 1983). Like glucagon, epinephrine acts within minutes and produces a transient increase in glucose production but continues to support basal rate glucose production thereafter. By contrast to glucagon, however, epinephrine also limits glucose utilization predominantly through the β-adrenergic mechanism (Rizza *et al.*, 1980; Berk *et al.*, 1985; Clutter *et al.*, 1988). Thus, in the event of hyperepinephrinemia, the persistent limitation of glucose usually causes hyperglycemia.

Another hormone that has been shown to have an effect on glucose levels is cortisol. Long term elevation (after 2-3 hours) in cortisol increases plasma glucose levels (Shamoon *et al.*, 1981). Pancreatic polypeptide is secreted by the pp-cells of pancreatic islet cells and has a hyperglycemic effect. The pancreatic content of this polypeptide is of a similar order of magnitude as that of glucagon and insulin. Yet another hormone that has a hyperglycemic effect is arginine vasopressin which increases in response to hypoglycemia.

The mechanisms of altered and defective counter-regulation of glucose in patients with IDDM have been reviewed by Cryer (1988) and Cryer *et al.* (1989). Absent or blunted glucagon secretory response to hypoglycemia and to physiological decreases in glucose are apparent in most IDDM patents. To the extent that glucagon secretory response are deficient such patients are dependent on epinephrine to promote hyperglycemia. Altered glucose counter-regulation and defective glucose counter-regulation seen in IDDM patient are the result of disease-related deficiency in glucagon secretory response and of combined defects in the glucagon and epinephrine secretory responses to hypoglycemia, respectively. Intensive insulin therapy of these individuals places them at a substantial risk of severe hypoglycemia.

Thus, glucagon plays a central role in promoting glucose recovery in hypoglycemia. Epinephrine compensates largely for deficient glucagon secretion. Glucose recovery from insulin-induced hypoglycemia fails to occur in the absence of both glucagon and epinephrine. Furthermore, even when the insulin is dissipated, glucose counter-regulation can become totally disrupted by combined deficiencies in glucagon and epinephrine.

2. Desired Properties of an Engineered Secretory Cell

Thus, in a particular embodiment, the present invention provides an immortal human neuroendocrine secretory cell that may be employed in the treatment, prevention or alleviation of hypoglycemia and/or diabetes. Such cells will be especially useful in singular or combination therapy of IDDM in which the glucose counter-regulatory system has been compromised. Thus, immortalized secretory cells that have a stable neuroendocrine phenotype will be used to provide a counter to the hypoglycemic effects of insulin by providing hyperglycemic factors or novel ways to inhibit insulin secretion. In particular embodiments, this will entail the expression and secretion of glucagon. In other embodiments, the present invention will entail the surface expression of α- or β-adrenergic receptors which will promote the hyperglycemic effects of epinephrine and epinephrine-related compounds, *e.g.*, norepinephrine on insulin-secreting cells.

The present invention will in particular aspects employ immortal human neuroendocrine cell lines to secrete hormones or express polypeptides that will be useful in glucose regulation and counter-regulation, in order to achieve a physiologically normal glucose concentration in disorders such as diabetes. In certain embodiments, cells will be engineered to be phenotypically like pancreatic α-cells in that they are glucagon secretory cells. Such cells may be engineered to express and secrete glucagon, or will have express receptors that will augment, increase or otherwise enhance the production of endogenous glucagon, in response to hypoglycemia. In other embodiments, cells will be engineered to be phenotypically like pancreatic β-cells. Such cells will express and secrete insulin, and regulators of insulin secretion, such as GLUT-2 and glucokinase. In a preferred aspects these cells will be engineered to express polypeptides that will suppress, decrease or otherwise down-regulate insulin production or action in response to hypoglycemia. In particular aspects the β-cells will have glucose sensing capacity in the form of GLUT-2 and glucokinase expression as described in U.S. Patent 5,427,940.

There are a number of properties that are desired of a cell line that will be representative of an immortal human neuroendocrine secretory cell for the purposes of the present invention. First, it is important to ensure that engineered cell lines have a high transgenic polypeptide content and secretory capacity. In particular embodiments, the cell can be a pancreatic β-cell or a cell that is a glucagon secreting cell. The regulated pathway of the β-cell encompasses both acute regulation of polypeptide secretion (*i.e.*, a large increment between the unstimulated and stimulated states) and the complete processing of polypeptide. In islet β-cells, secretory granules allow the storage of insulin as a depot at the plasma membrane that can be released within seconds of arrival of a fuel-derived or hormonal signal, and also serve as the site of conversion of proinsulin to insulin by virtue of their high concentrations of the relevant convertases PC1 (also known as PC3) and PC2. The presence of secretory granules and retention of proinsulin processing capacity represent a major advantage of insulinoma and other neuroendocrine cell lines relative to cells less specialized for secretion of peptide hormones such as hepatoma cells or fibroblasts.

A second parameter is that the cell may be equipped with a capacity for secretagogue sensing and responsiveness. For example, the cells able to sense the glucose concentration of their environment are preferred. U.S. Patent 5,427,940 (incorporated herein by reference) described engineering this capacity into cells.

Finally, it is imperative that engineered cell lines retain phenotypic and genotypic stability. This includes both genes that are inserted or deleted during the course of engineering and key endogenous genes. As discussed herein, this is a significant limitation on current technology.

3. Host Cells

A variety of host cells are contemplated to be useful as the starting cells that will be engineered to provide the therapeutic expression of the polypeptides discussed herein. In the case of secreted polypeptides such as glucagon it will be desirable, for the polypeptide to be released from cells in response to the hypoglycemic environment of the cell. In the case of insulin secreting cells, it will be desirable to provide these cells with glucose and counter-regulatory sensing capacities such that in hypoglycemic conditions the insulin secretion can be down-regulated. In those embodiments in which the polypeptide is a receptor, it will be desirable to have the receptor expressed at the cell surface, such that the agent acting through said receptor can act to lower insulin levels in the case of a hypoglycemic environment.

The immortalized human neuroendocrine cells and cell lines that retain the characteristics of the primary cell, as provided with the instant invention, are the preferred starting material. The instant cells and cell lines overcome the failures of previous attempts at immortalization of human pancreatic β-cells, which have resulted in cell lines that do not retain the defining properties of the primary β-cell, such as the capacity to synthesize insulin and secrete it from the regulated secretory pathway.

Engineering of cells to synthesize proteins for the purposes of the present invention will make use of many attributes of these cells. Regulated secretory cells present a natural bioreactor containing specialized enzymes involved in the processing and maturation of secreted proteins. These processing enzymes include endoproteases (Steiner *et al.,* 1992) and carboxypeptidases (Fricker, 1988) for the cleavage of prohormones to hormones and PAM, an enzyme catalyzing the amidation of a number of peptide hormones (Eipper *et al.,* 1992). Similarly, maturation and folding of peptide hormones is performed in a controlled, stepwise manner with defined parameters including pH, calcium and redox states.

Complete processing requires sufficient levels of the processing enzymes as well as sufficient retention of the maturing peptides. In this way, physiological signals leading to the release of the content of the secretory granules ensures release of fully processed, active proteins. This is important for both maximum production for *in vitro* purposes, and for the use of cells for *in vivo* purposes.

A cell specialized for secreting proteins via a regulated pathway also can secrete proteins via the constitutive secretory pathway. Many cell types secrete proteins by the constitutive pathway with little or no secretion through a regulated pathway. As used herein, "secretory cell" defines cells specialized for regulated secretion, and excludes cells that are not specialized for regulated secretion. The regulated secretory pathway is found in secretory cell types such as endocrine, exocrine, neuronal, some gastrointestinal tract cells and other cells of the diffuse endocrine system.

The origin of the starting cells for use in the present invention thus include human tissues and tumors of neuroendocrine lineages that have a well defined regulated secretory pathway. Cells with defined conditions for culturing *ex vivo* with some replicative capacity are also preferred. Neuroendocrine cells, such as pancreatic β cells, pancreatic α-cells and pituitary cells are preferred for use in the present invention. Examples of such cells are shown in Table 1 (Pearse and Takor, 1979; Nylen and Becker, 1995).

The neuroendocrine cells of the invention will preferably secrete one or more of the endogenous secretory polypeptides listed herein in Table 1. Stable α cells that secrete glucagon will be preferred in certain aspects of the invention, with cells that secrete correctly processed human glucagon being more preferred. Also preferred will be those cells that express receptors for providing control of glucagon secretion. The stable β-cells of the invention also may advantageously be engineered to express α2-adrenergic receptors, pancreatic polypeptide receptors, vasopressin receptors, glucocorticoid receptors and the like. Of course, for therapeutic purposes, engineered β-cells will further express insulin as well as glucose sensing genes such as GLUT-2 and glucokinase as described in U.S. Patent 5,427,940.

In addition to pancreatic β-cells, pancreatic α-cells also are preferred for use in the present invention. These cells will be engineered for glucagon expression and secretory capacity into the starting cells disclosed herein above. Alternatively, the endogenous glucagon secretion of these α-cells will be augmented using stimulation by epinephrine and epinephrine related molecules such as norepinephrine and clonidine. Such epinephrine stimulatory phenotype may be engineered into the α-cell by providing the cell with the ability to express an adrenergic receptor. More particularly the cell will express an α-adrenergic receptor, in even more preferred embodiments the cell will express an α1-adrenergic receptor.

The term "glucose counter-regulation" is a term that refers to the effects on the glucose metabolism that are opposite to those mediated by insulin. As such glucose counter-regulation refers to the correction of hypoglycemia or the prevention of hypoglycemia. Thus, a factor that mediates glucose counter-regulation reduces insulin secretion and raises the glucose level from hypoglycemic toward hyperglycemic, but more preferably towards the normal physiological glucose levels.

A glucose counter-regulatory hormone is defined as one that responds to hypoglycemia either with alterations in the plasma levels or activity. Most often it is envisioned that such hormones will increase in levels or activity in response to hypoglycemia. These hormones can include those known to be directly glucose counterregulatory such as epinephrine, glucagon, pancreatic polypeptide, vasopressin, and cortisol, and also includes hormones that are glucose counterregulatory by indirect mechanisms such as growth hormone, various neuropeptides, ACTH, somatostatin, and so on. It is also within the scope of this invention to engineer secretory functions into cells for novel mechanisms of glycemic control. For example, the effect of overexpression of various receptors and/or channels on insulin-secreting cells may improve the regulation of secretion to minimize inappropriate release of the peptide under hypoglycemic conditions. Any receptor or channel that affects membrane polarization, especially to hyperpolarize, will impact secretory function.

The list of potential proteins that will impact secretory function include the following: alpha-2 adrenergic receptor (ATCC number 59303, HPalpha2GEN Genbank accession numbers M18415, M23533, incorporated herein by reference), glucagon-like peptide I receptor (Genbank accession numbers: L23503, U10037, U01156, U01104: each incorporated herein by reference), somatostatin receptor V (mouse Genbank accession number AF004740; human Genbank accession numbers: L14865, L14856, M81830, M96738, M81829, L07833 each incorporated herein by reference), SUR channel (Genbank accession numbers L78207, U63455, L78243, incorporated herein by reference), KIR channel, pancreatic polypeptide receptor (Genbank accession numbers: Z66526, U42387, U42389 each incorporated herein by reference), muscarinic receptor (Genbank accession numbers: X52068, X15264, X15265, X15266, AF026263 each incorporated herein by reference); glucocorticoid receptor (Genbank accession numbers: M10901, M11050 each incorporated herein by reference), human (glucose-dependent insulinotropic peptide) GIP receptor (Genbank accession number X81832, incorporated herein by reference) human PACAP/VIP receptor (Genbank accession numbers L36566, D17516, U18810, each incorporated herein by reference) human β-cell type Ca2+ channel (Genbank accession number M83566 incorporated herein by reference) and leptin receptor (Genbank accession numbers: U43168, U52912, U52913, U52914 each incorporated herein by reference). In certain embodiments, the cells of the present invention may be employed to screen for other such compounds.

Glucose is the most important stimulator of insulin secretion, not only because of its potent direct effects but also because it is permissive for the stimulatory action of a wide array of other secretagogues. While there is good evidence to suggest that glucose exerts its effect through its own metabolism, resulting in the creation of signals that appear to work through modulation of ion channel activities and influx of extracellular Ca^{2+}, the exact nature of the metabolic coupling factors remains unknown. The magnitude of the insulin secretory response appears to be related to the rate of β-cell glucose metabolism, and both parameters are sharply increased in response to modest increments in extracellular glucose concentrations within the physiological range of 4 to 8 mM. β-cells are equipped with the glucose transporter GLUT-2 and the glucose phosphorylating enzyme glucokinase which have kinetic properties, particularly a relatively low affinity for glucose, that are ideal for modulation of glucose responsiveness at the relatively high concentrations of the sugar encountered in the circulation (Newgard and McGarry 1995).

E. *In vivo* Drug Screening Assays

In addition to the *in vitro* methods for drug screening discussed in detail in **Section XII(B)** above, the present invention also provides for the testing of candidate substances for their ability to modulate secretory function of cell in *in vivo* contexts. This approach has the added advantage of assessing (i) the function of cells under normal, physiologic conditions including the presence of various intercellular signaling mechanisms and (ii) the ability of candidate substances to target cells local, regional or distal to their site of administration and (iii) localization and/or tissue distribution of a secreted metabolite or downstream effector metabolite. Different formulations including time release compositions also may be assessed. Finally, this format permits testing on the basis of physiologic states rather than the mere increase or decrease of secretory function. This provides additional information on the actual potential therapeutic benefit of the substance for the host, testing of therapeutic vs. toxic concentrations to establish therapeutic ranges and drug safety parameters, as well as allowing for *in vivo* interactions to be monitored.

The preferred embodiment for *in vivo* screening of candidate substances involves the use of a nude rodent model. The nude mouse lacks immune functions that might compromise or interfere with testing of implanted cells of the present invention. This system is well characterized and is used for a variety of other purposes including a model of transplanted human cancer. Yet another preferred model is one in which the animal has diabetes (IDDM or NIDDM).

Briefly, immortalized secretory cells of the present invention will be transferred, as part of an implantable device (described elsewhere in this document) into a suitable site of the host animal. Typically, subcutaneous implant on the flank, back or hindlimb of the animal, or intraperitoneal insertion, is preferred. Intramuscular implant, usually on the hindlimb, also is contemplated. Particular issues that will affect the choice of implantation site include (i) similarity to the normal site the cells might be found or implanted clinically and (ii) the importance of establishing a supporting vasculature structure for the implant.

After a suitable period of time for stabilization of the implant, usually 1 to 10 days, the animal is ready for testing. The initial step involves the determination of steady state levels of any metabolite that will be used as a read-out for effects on modulation of the implanted cells. This often will involve taking peripheral blood measurements of the metabolite of interest. Such metabolites include, but are not limited to, glucose, insulin, glucagon, GLP-1, amylin, leptin, somatostatin and growth hormone. Alternatively, one may assess functional attributes of the animal such as alteration in blood glucose in the case of insulin and loss of body fat in the case of leptin. Other parameters that could be monitored include, in body weight, food intake, blood pressure, metabolic rate, body temperature, serum minerals, *etc.*

Once steady state levels and conditions have been determined, the candidate substance is administered. Depending on the location of the implant and the particular purpose for the assay, the candidate substance, formulated in a pharmacologically acceptable fashion, will be administered to the animal. Suitable routes include oral, rectal, vaginal, buccal, topical, inhalation or intravenous or intraarterial injection. Also contemplated are intramuscular, intraperitoneal, intraocular, subcutaneous or submucosal administration.

As stated above, the metabolite will be tested from the appropriate tissue or fluid from the host animal. Fluids include blood, lymph, saliva, sputum, feces, urine, semen or tears. Tissues that may be sampled include liver, brain, muscle, pancreas, spleen, testis, ovarian, stomach, intestine, endocrine glands, adrenal glands and kidney. Depending on the metabolite, different methodology, also described above, will be used to separate and identify the presence, quantity and/distribution of the metabolite. In addition, histologic examination, involving microscopy, may be performed. Modulation of the metabolite or function, in the presence of the candidate substance, as compared with the levels determined prior to provision of the candidate substances, will indicate that the candidate substance is a modulator of that metabolite or function.

Following completion of the experimental phase, rescue of the implant may be performed for the purpose of determining the secretory status of the implant cells. Any change in the behavior or characteristics of the cells could impact the results. Proper controls will include animals implanted with empty devices and animals implanted with devices populated with "placebo" (non-responsive, non-secretory) cells.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Regulated Secretory Pathway in βG H03 Cells

βG 498/20, βG 498/44, and βG 498/45 were studied for the capacity to secrete insulin from the regulated pathway and respond to modulators of secretion. Cells were plated in 12-well tissue culture dishes (250,000 cells/well), maintained for 72 hr in BetaGene Medium, and washed twice, 20 min each, in HEPES/bicarbonate-buffered salt solution (HBBSS; in mmol/l: 114 NaCl, 4.7 KCl, 1.21 KH_2PO_4, 1.16 $MgSO_4$, 25.5 $NaHCO_3$, 2.5 $CaCl_2$, 10 mM HEPES) supplemented with 0.1% BSA but lacking glucose. Insulin secretion was stimulated by incubating the cells for 2 hr in HBBSS containing 0.1% BSA and supplemented with 10 mM IBMX, 100 μM carbachol, or 10 nM of the phorbol ester, PMA; all in the presence or absence of 10 mM glucose.

As shown in FIG. 2A, βG 498/20 respond robustly to carbachol and PMA (about 10- to 15-fold over basal), however, the cells were unresponsive to glucose and IBMX. βG 498/44 and βG 498/45 were nearly identical in their secretion profiles as compared to βG 498/20. These data are consistent with the presence of a regulated secretory pathway; and it appears that protein kinase C-mediated events dominate in the regulation of secretion. However, as expected, these lung neuroendocrine cell lines do not mimic the response of pancreatic β-cells or β-cell lines to glucose alone or the glucose-potentiator, IBMX.

βG 498/45 was further engineered for increased levels of insulin expression by the introduction of a number of plasmids, all of which encoded human insulin but varied in the genes encoding antibiotic resistance. The 793, 794, and 796 cell lines are resistant to mycophenolic acid, puromycin, and hygromycin, respectively. The data in FIG. 2B show the presence of a regulated secretory pathway in the progenitor cell line (498/45) and the maintenance of this capacity through a second round of engineering (793, 794, and 796 cell lines). Insulin content and secretion were increased by about 3- to 4-fold in second generation clonal cell lines. The insulin secreted from two of these high-producing clones (793/28 and 793/15) was fractionated by high-performance liquid chromatography, and immunoreactive insulin species were quantified by radioimmunoassay using human insulin standards.

Proinsulin was effectively processed to mature insulin, with mature insulin representing the majority of the total insulin in media extracts.

EXAMPLE 2

High Levels of Transgene Expression in βG H03 and βG H04 Cells

In addition to human insulin, βG H03 cells have been engineered to express glucagon like peptide 1 (GLP-1) and human growth hormone (hGH). The former peptide was efficiently processed from the precursor pre-proglucagon and amidated. Clonal cell lines capable of secreting 1 ng GLP-1/million cells/ 24 hr were isolated.

The human neuroendocrine cell line βG H04 was stably transfected with BetaGene plasmid AA603 (SV40 driving the expression of neomycin phosphotransferase and CMV driving expression of human insulin) resulting in monoclonal cell lines βG 707/55, 707/63, 707/76, 707/94 and 707/96. The clonal cell lines were studied for their ability to secrete insulin in response to various modulators of secretion, as previously described. In each of the 5 clonal cell lines insulin secretion did not change with respect to basal in response to stimulation by 10 mM IBMX, 100 mM carbachol, or 10 nM PMA and 10 mM glucose.

In a subsequent secretion study, the cells were compared at basal (0 mM) and stimulated (25 mM KCl + 2.5 mM Forskolin + 50 mM IBMX) conditions in HBBSS. A high concentration of KCl causes cell membrane depolarization and a subsequent release of all peptides destined for secretion. Forskolin and IBMX enhance the cascade by increasing the production of cAMP, thereby stimulating secretion. Thus, this combination of secretagogues should cause the cells to void any peptides stored in secretory granules. FIG. 3 illustrates the secretion response of βG 707/55, 63, 76, 94, 96 clones and the clonal derivative of βG H03 (βG 498/45) to the secretagogue cocktail described above. As expected, βG 498/45 cells, secrete in excess of 500 ng/flask/hour of insulin. In contrast, βG 707 clonal lines secrete a negligible amount of insulin under these conditions. Cell content of βG 707/55 was analyzed by HPLC for insulin. A small proinsulin peak was detected, however no mature insulin was detected within these cells.

In a separate stable transfection of βG H04 cells, BetaGene plasmid CD303 (CMV driving expression of human growth hormone, SV40 driving neomycin resistance) was used to establish cell lines resistant to G418. The monoclonal cell line βG 785/5 was analyzed for cell content versus secreted human growth hormone on a Western blot. The results indicated a small fraction of human growth hormone stored within the cells and a large fraction of this peptide in the medium.

These data suggest that the βG H04 cell line, despite the presence of multiple proteins associated with a neuroendocrine phenotype, is not a preferred candidate for secretion of transgenic peptides from the regulated secretory pathway. These cells use a constitutive mode of secretion, rather than a regulated secretory pathway, perhaps due to an inability to depolarize the cell membrane or an absence of dense core granules for peptide storage. Several factors controlling peptide trafficking may also be missing in these cells, further complicating regulated peptide release. In addition to falling short of the regulated peptide secretion requirements, the βG H04 cells do not process insulin to its mature form. Unprocessed proconvertase 1/2 (PC1/PC2) is present in this cell line, and only proinsulin is detected by HPLC. These data highlight the inefficiencies of neuroendocrine cells with respect to creating a β-cell phenotype. Although this cell type displays many desirable factors needed to mimic the β-cell, it may also display many undesirable characteristics, emphasizing the need to thoroughly study and analyze each candidate neuroendocrine cell line.

In order to express high levels of a processed peptide hormone from clonal derivatives of the βG H04 cell line, it may be necessary to create fusion proteins containing a furin site between a given prohormone sequence and the sequence encoding the mature peptide hormone. Such a site may provide the capacity for processing of the transgenic fusion protein through the constitutive pathway that apparently dominates secretion from these cells. Alternatively, the overexpression of PC1 and/or PC2, proteins involved in granule formation such as chromagranin A and chromagranin B, or proteins required for trafficking through the regulated secretory pathway such as carboxypeptidase E may be required for restoration of a functional regulated secretory pathway in the βG H04 cell line. Carboxypeptidase E is a particularly attractive candidate, as carboxypeptidase E is not expressed in βG H04 cells.

EXAMPLE 3

Methods for Creating a Human β-Cell Line

As described in the previous examples, there may be potential drawbacks for the use of existing human neuroendocrine cell lines for glucose-regulated delivery of human insulin. For this reason, a two-step transformation procedure has been devised to create a human β-cell line (FIG. 4). Preferred starting materials consist of either a surgically removed human neuroendocrine tumor such as an insulinoma, or isolated primary tissue such as human islets. The β-cells in these tissues proliferate at a very slow rate, therefore, the first step is to get them to grow. This is accomplished by infecting insulinomas and/or islets with a recombinant adenovirus expressing an oncogene under the control of the rat insulin 1 gene promoter (RIP). Adenovirus is the preferred viral vector because it infects and expresses the transgene in nondividing cells. RIP selectively expresses the oncogene, in this case, temperature sensitive SV40 T-antigen (tsTAG), in only β-cells. In response to tsTAG expression, the β-cells proliferate while other cell types remain quiescent. The adenovirus does not integrate into the genome and thus advantageously does not provide long-term expression of tsTAG. Following this initial transformation, a second transformation step is required.

The second step is to immortalize the proliferating β-cells by infection with a recombinant retrovirus also expressing an oncogene like tsTAG under the control of RIP. Retroviruses require cellular division in order to integrate into the genome. Once integrated, the transgene is stably expressed resulting in an immortalized cell.

EXAMPLE 4

Culturing of Human Insulinomas

The present invention contemplates the use of cell lines derived from human insulinomas as starting cells for the instant methods to produce immortalized human neuroendocrine cells. This example describes the culturing of human insulinomas.

Patients with insulinomas are treated by surgical excision of the tumor. At the time of excision, the tumor tissue is immediately placed in sterile, tissue culture medium (BetaGene medium supplemented with 3.5% fetal bovine serum (FBS), 200 U penicillin/streptomycin, and 50 μg/ml gentamycin). The tissue is kept on ice and sterile, keeping the transit time to less than 30 minutes. Using sterile techniques, the tissue is minced with iris scissors until it is reduced to pieces 1 mm^3 or smaller. The tumor is then transferred to 40 mesh tissue sieve through which the large pieces are forced using rubber pestle. The cells are then washed twice for a period of 15 minutes each with fresh culture media containing antibiotics.

The tissue is then split onto standard Falcon tissue culture dishes and dishes coated with matrigel extracellular matrix. The tissue is maintained under standard tissue culture atmospheric conditions of 37°C; 5% CO_2/95% air; and humidified. The tissue is then cultured with media composed of 30% conditioned tissue culture media (BetaGene medium containing 3.5% fetal bovine serum (FBS) conditioned by culture with βG 261/13, a rat β-cell line stably transfected with pCB6 (CMV promoter and neo resistance marker) expressing the full length human growth hormone coding region), 70% BetaGene Medium, 1% FBS, 50 μg/ml gentamycin. To prevent loss of unattached cells, only 75% of media is replaced by removing old media from the top of the dish. Using this approach a human insulinoma (HT6#2) was cultured, and has been found to secrete insulin for over 150 days (FIG. 5).

EXAMPLE 5

Human Islet Function in BetaGene Medium

Human islet preparations were obtained from the distribution center of The Diabetes Research Institute, Miami FL. The volume of islets received are expressed in islet equivalents (IEQ). An islet equivalent is the number of cells/volume that is found in an islet with a diameter of a 150 µm. Insulin content and secretory response of the islets were assayed first upon receipt and second after culture in BetaGene medium. Proper insulin processing was also analyzed before and after culture in BetaGene medium.

A. Receipt and Preparation of Islets

Islet prep suspensions were spun down in a bench top centrifuge at 1000 rpm for 2 minutes at room temperature to pellet the cells. The shipping medium was aspirated leaving approximately 5 ml behind to avoid disrupting the pellet. The pellets were resuspended in the remaining 5 ml medium and transferred to a new 50 ml conical tube. About 40 ml of BetaGene medium supplemented with 2% fetal bovine serum, 500 mg/ml gentamycin, 200 units/ml penicillin, and 200 mg/ml streptomycin was added to each suspension and allowed to incubate at room temperature for 15 minutes. The samples were spun down a second time, all but 5 ml of the medium was aspirated, and a fresh aliquot of BetaGene medium with supplements was added and allowed to incubate for another 15 minutes. After the second and final incubation, the islets were spun down and all of the medium was removed. The pellet was resuspended in complete BetaGene medium at a density of 1000 IEQ per milliliter.

B. Alginate Encapsulation of Human Islets

The study of islets in long term culture is facilitated by encapsulating the cells in alginate. Islet cells do not divide in culture and may be overrun by various replicating cells which are present in islet preps as shipped. Encapsulating the cells immediately upon receipt minimizes the growth of fibroblasts and other cell types.

The islets were resuspended in a 2% sodium alginate solution (50% high viscosity and 50% low viscosity sodium alginate made up in complete BetaGene medium) at a concentration of 1000 IEQ per 1 milliliter of alginate. The suspension was transferred to a syringe and allowed to sit at room temperature for 5 minutes to allow all air bubbles to rise to the surface. A 25 gauge needle was attached to the syringe and the islet/alginate slurry was dispensed through the syringe into a 50 ml conical tube containing approximately 35 ml of 1.35% $CaCl_2$/20 mM HEPES. Beads were formed as the slurry hit the surface of the $CaCl_2$ solution, and were completely polymerized after about 10 minutes. The $CaCl_2$ solution was removed carefully and the beads were washed with two volumes of BetaGene medium/20 mM HEPES. The encapsulated islets were then cultured with the medium under conditions described for each study.

The glucose concentration of BetaGene Medium was based on the glucose concentration that was provided 50% maximal stimulation during acute secretion studies. Initial studies (n=5 independent islet preparations) indicated that 50% maximal stimulation of human islets was provide by a medium glucose concentration of 7.4 ± 0.2 mM. The glucose concentration of unmodified BetaGene Medium was manufactured at 7.8 mM glucose (or 140 mg/dl).

3. Insulin Content and Processing of Human Islets

A portion of each islet preparation was used to assess insulin content of the islets upon receipt. Prior to culture in BetaGene medium, 2000 IEQ were removed from the stock and spun down to pellet the islets. The medium was removed completely without disturbing the cell pellet. The islets were washed one time with phosphate-buffered saline (PBS) and spun down. The pellet was dispersed in 0.5 ml content buffer (1M acetic acid, 0.1% BSA) and frozen at -80°C. The cells were thawed, sonicated (3 bursts at setting 5-6 on a Fisher Scientific 60 sonic dismembrator) and the insoluble debris were pelleted at 14,000 rpm for 10 minutes at 4°C. The supernatant was then transferred to a clean tube and a portion was analyzed by HPLC.

Encapsulated islets were cultured in BetaGene medium and fed 3 times weekly. The islets were removed from the alginate to extract the insulin content. To recover the islets, the beads were incubated in 6 mM EDTA/10 ml BetaGene medium and the alginate was dispersed by pipetting until the mixture became homogeneous. The mixture was centrifuged at 1/2 speed in a benchtop centrifuge for 5 minutes, the supernatant with alginate was removed and the islets washed with 10 ml PBS/2 mM EDTA. The solution was spun again and the pellet was resuspended in 5 ml PBS to remove EDTA, spun again and resuspended in content buffer for analysis by HPLC as described above.

4. Glucose Dose-responsive Secretion

Alginate encapsulated islets were cultured in 24 well plates for at least 4 days, with about 50 IEQ/well (or 5 beads) in 2 ml of medium. The day before the study the culture medium was replaced with fresh medium. The day of the study the islets were equilibrated for 90 minutes with BetaGene Medium with low glucose. The medium was then removed and replaced with 1 ml of RPMI without glucose, or Modified BetaGene Medium, (manufactured without glucose), that was supplemented with glucose to provide concentrations between 2.2 and 22 mM glucose, and 22 mM + IBMX. The islets were then incubated at 37°C for 90 minutes and samples collected at the end of 90 minutes for assay of insulin. Each experimental value usually represents results from 6 replicate wells. The glucose concentration providing 50% of maximal stimulation (Stim-50) was calculated from the fitted line of the glucose dose-response curve.

5. Cultured Human Islets and Insulin Secretion

Results in the literature indicate that culture of human islets with high glucose (11-22 mM) is deleterious to secretory function. However, others have indicated that the effects of high glucose were dependent on culture medium used. Culture (> 2 weeks) of human islets was reported to result in a progressive loss of glucose-responsive insulin secretion over 2 weeks. Different media or glucose concentrations slowed, but did not prevent, this loss. The glucose concentration of BetaGene Medium was based on the concentration that gave 50% of the maximal glucose-induced response. The effect of different glucose concentrations were tested to ensure that a medium glucose concentration based on 50% stimulation was appropriate. Islets were cultured in BetaGene Medium with 3.9 mM, 7.8 mM and 22 mM glucose for about 2 weeks. Although lower glucose was less deleterious than the higher concentration, both resulted in impaired secretory response (FIG. 6). The results demonstrate that neither lower nor higher concentrations of glucose provide improved performance. These results confirm that 7.8 mM glucose in BetaGene Medium is sufficient to sustain secretory function of human islets.

The serum requirements of human islets were tested in long term (about 2 months) cultures supplemented with various amounts of serum, 1%, 3.5%, or 10% FBS and 5% horse serum (ES). In four independent isolations the average daily insulin output for 60-90 days was minimally affected by amount of serum supplementation. However, the overall tendency was for higher FBS to yield lower output. Similarly, in an acute secretion experiment, insulin secretion from islets cultured in 10% FBS exhibited lower response to glucose or to a stronger mixed secretagogue stimulus (FIG. 7). The sustained insulin output from human islets with 1% FBS supplementation (in BetaGene Medium) suggested that human islets may also secrete insulin and survive under serum-free conditions.

In order to compare the effect of different media on human islets, the insulin output over 2-3 months was studied without serum. Islets were cultured with BetaGene Medium, Medium 199, alpha MEM, and CMRL, all with equivalent glucose, and 0.1% BSA. In all four isolations the insulin output was the highest with islets cultured in BetaGene Medium (FIG. 8). In fact, the average insulin output of BetaGene Medium without serum-supplementation was not markedly different from cultures, of the same isolation, cultured with 3.5% or 10% FBS (average serum-free with 4 isolations studied was 112% of cultures supplemented with 3.5% and 10% FBS). Many transplant surgeons consider CMRL the medium of choice for use with human islets (Ricordi *et al.*, 1988). However, CMRL performed the poorest, with essentially no islet survival past 2 months with all 4 isolations studied.

The capacity of BetaGene Medium to sustain the dose-responsive nature of the insulin secretory response was evaluated with continuous cultures. Human islets were stimulated with varied glucose concentrations at intervals to monitor secretory changes that may occur with time. It has been previously noted that the capacity of human islets to respond to glucose is impacted by isolation methods and conditions, in particular, cold ischemia time. Cold ischemia of the preparations studies varied between 10 and 22 h. Variables related to donors and isolations produce considerable variation among islet isolations. As a result, the magnitude of response is not found with all preparations. However, a common finding was an initially poor response, with increased function with time of culture in BetaGene Medium, and a maintained capability to secrete insulin in response to glucose for times >4 months (FIG. 9).

The sustained secretory function for months in culture was also accompanied by maintained insulin content and insulin processing. This is illustrated with both islets that have initially low or initially high insulin contents, and with islets that initially exhibit minimal insulin processing capacity. The insulin content of islets from HI28 was low upon arrival, 0.3 μg/1000 IEQ with >90% mature, processed insulin. The islets secreted in response to glucose, with 50% stimulation at 7.5 mM. The insulin content of mature, processed insulin with HI28 islets cultured 1.5 months in BetaGene Medium was increased 4 fold to 1.3 μg/1000 IEQ. An islet preparation, HI26, with an initially high insulin content of 5.0 μg/1000 IEQ, and 94% mature insulin, was cultured long term. The insulin content of these islets were maintained for 72 d of culture with BetaGene Medium. The final insulin content was 4.97 μg/1000 IEQ, with 89% mature, processed insulin.

Finally, culture in BetaGene Medium restored processing with 2 islet isolations that initially had almost no mature insulin. FIG. 10A shows the fractionation of insulin extracted from islets of HI21. Initially, 99% of the insulin was unprocessed insulin, with only 29 ng mature insulin/1000 IEQ. The mature insulin content was increased 18-fold to 512 ng/1000 IEQ after 4 weeks of culture in BetaGene Medium; this represents >90% of the insulin content (FIG. 10B). Another islet preparation initially making only proinsulin upon arrival was HI27. Prior to culture, essentially 100% of the insulin content was proinsulin. HI27 islets were cultured 8 weeks, and then insulin content was fractionated by HPLC. With this isolation as well, islets had regained the capacity to process insulin. In both of these preparations, while insulin processing improved the total insulin content (mature + unprocessed insulin) was decreased.

These data demonstrate that human islets cultured in BetaGene medium exhibit improved secretory function, maintained glucose-responsiveness, while maintaining or even increasing proteolytic processing of insulin and insulin content.

EXAMPLE 6

Expression Plasmid Constructs for Enhanced Proliferation and Immortalization

A temperature-sensitive mutant of the SV40 large T antigen, tsA58 (Bourre and Sarasin 1983) was isolated from pBS/tsA58. The tsA58 coding region (tsTAG) was isolated by partial digestion with *Hpa*I, treatment with Klenow fragment, followed by digestion with *Eco*R1. The resulting 2532 base fragment (SEQ ID NO: 1) was ligated into pCMV8/IRES/Neo (Clark *et al.*, 1995) previously digested with *Bam*H1, Klenow treated, then digested with *Eco*RI. The resulting expression plasmid, pCMV/tsTAG/IRES/Neo, expresses a bicistronic message driven by the human cytomegalovirus promoter with the tsTAG upstream of the G418 resistance gene. Drug resistance to G418 results from translation of the downstream Neo gene due to the internal ribosome entry site (IRES, Macejak and Sarnow, 1991). A second tsTAG expression plasmid was constructed in which the CMV promoter was replaced with the rat Insulin 1 promoter (RIP). pCMV/tsTAG/IRES/Neo was digested with *Spe*I and *Eco*RI, removing the CMV promoter, and replaced with RIP on a 440 bp *Spe*I/*Eco*RI fragment derived from pRIP7/INS (Clark *et al.*, 1996), generating pRIP/tsTAG/IRES/Neo.

Recombinant adenoviruses expressing tsTAG under the control of either the RIP promoter or the CMV promoter were constructed. The tsTAG encoding fragment was isolated from pCMV/tsTAG/IRES/Neo by digestion with *Sal*I, treatment with Klenow fragment, followed by *Eco*RI digestion. The fragment was ligated into pAC/RIP that had been digested with *Bam*HI, Klenow treated and digested with *Eco*RI, generating pAC/RIPtsTAG. pAC/CMVtsTAG was constructed by removing tsTAG from pBS/tsA58 and ligating into pAC/CMV to produce pAC/RIPtsTAG.

293 cell culture and generation of recombinant adenovirus stocks, conditions for adenovirus stocks, as well as conditions for adenovirus infection of cells are done as described (Becker *et al.*, 1994b; incorporated herein by reference).

Retroviral expression plasmids were constructed in order to produce recombinant retroviruses capable of expressing tsTAG under the control of the tissue-specific rat insulin promoter. A fragment containing RIP/tsTAG was isolated from pRIP/tsTAG/IRES/Neo by digestion with *Sal*I, Klenow treatment followed by *Spe*I digestion. This fragment was ligated into pBS/hGH PolyA that had been treated with *Xba*I, Klenow treated and digested with *Spe*I, generating pBS/RIP/tsTAG/hGH PolyA. The hGH PolyA sequence in pBS/hGH PolyA is a 625 base sequence which directs efficient transcriptional termination and polyadenylation of mRNAs. Finally, pBS/RIP/tsTAG/hGH PolyA was digested with *Sac*I, Klenow treated, followed by digestion with *Sal*I allowing isolation of a RIP/tsTAG/hGH PolyA containing fragment. This fragment was ligated into two retroviral plasmids, pBabeNeo and pBabePuro (Morgenstern and Land 1990), following digestion with *Sna*BI and *Sal*I, generating pBabeNeo/RIPtsTAG and pBabePuro/RIPtsTAG, respectively. Additionally, the same *Sac*I, Klenow treated, *Sal*I digested fragment from pBS/RIP/tsTAG/hGH PolyA was ligated into pXT1 (Stratagene, Inc.) that had previously been digested partially with *Sal*I, Klenow treated, then digested with *Xho*I, generating pXT1/RIPtsTAG. Rat insulin promoter driven transcription of tsTAG is in the opposite orientation with respect to the retroviral LTR in all three plasmids. Several packaging cell lines for production of recombinant retroviruses are available (Miller and Buttimore, 1986; Danos and Mulligan, 1988; Miller, 1992).

An alternate approach to ensure that tsTAG is driven by the RIP promoter and not by the viral LTRs is to replace the normal untranslated region (UTR) of the retrovirus with a mutated UTR (von Melchner *et al.*, 1990; GenBank accession numbers M33167 through M33172, inclusive), which results in the loss of promoter/enhancer activity of the retroviral LTR. The mutated UTR strategy has been used previously for promoter trapping (von Melchner *et al.*, 1990). In the present invention, the inventors contemplate using this technique to confer specificity to the RIP promoter incorporated into the mutated retrovirus.

In addition to tsTAG, two more immortalization genes, the human papilloma virus 16 E6/E7 genes (Genbank accession number K02718) were cloned into the viral vector backbone LXSN (Miller and Rosman, 1989). E6/E7/LXSN was then introduced into the PA317 packaging cell line (Osborne *et al.*, 1990) to produce replication-defective recombinant retrovirus.

The full length IGF-1 receptor mRNA (Genbank accession number: X04434) was reverse transcribed and amplified by the polymerase chain reaction (RT-PCR). Total RNA was isolated from A549 cells using RNAzol B RNA isolation reagent (Cinna/Biotex Laboratories International). RT-PCR was performed using SuperScript™ Preamplification System (Life Technologies) followed by amplification with High Fidelity Platinum Taq polymerase (Life Technologies). One microgram of total RNA was transcribed at 42°C followed by 35 rounds of amplification with denaturation at 94°C (30 sec), annealing at 55°C (30 sec) and extension at 68°C (4.5 min) using oligonucleotides AT242; 5' GAGAAAGGGAATTCCATCCCAAATA 3' (SEQ ID NO:12) and AT249; 5' TTCAGGATCCAAGGATCAGCAGG 3' (SEQ ID NO:13). The IGF1 receptor cDNA was gel purified and cloned as an *Eco*RI/*Bam*HI fragment into *Eco*RI/*Bam*HI digested CW102 resulting in plasmid DM202.

EXAMPLE 7

Cell-Specific Expression of tsTAG and β-galactosidase

Human islet preparations and insulinomas contain many other cell types besides β-cells, therefore, it is important to target oncogene expression to β-cells. The use of RIP or a modified RIP promoter (discussed in Example 8) linked to an oncogene like tsTAG should target expression solely to β-cells as long as there is not promoter interference from viral promoters like retroviral LTRs. LTR interference could result in expression of tsTAG in other cell types besides β-cells, thus creating a more difficult task to isolate an immortalized β-cell line.

A. Stable Transfection of tsTAG

Cell-specific transcription of tsTAG was determined for both pBABE/Neo/RIPtsTAG and pXT1/RIPtsTAG (construction of these plasmids is detailed in Example 6) in RIN cells and in 293 human fibroblast cells. The retroviral plasmids were stably transfected into both cell types, and levels of tsTAG mRNA and protein were determined by Northern and Western blotting, respectively. Significant levels of tsTAG mRNA and protein were detected in RIN cell extracts containing either retroviral plasmid, whereas no expression of tsTAG mRNA or protein was observed in 293 cell extracts containing either retroviral construct. Temperature sensitivity of tsTAG was also observed in RIN cells as significantly more tsTAG protein was produced at the permissive temperature of 33.5°C than at the nonpermissive temperature of 37.0°C. This result corroborates the findings detailed by Frederiksen *et al.* (1988) in which high-level expression of tsTAG was observed by immunostaining at 33.0°C, but almost no expression was observed at 39.0°C. Because there was no tsTAG present in 293 cells, these results confirm that the viral LTR is not interfering with RIP to express tsTAG in non-β-cells.

B. Viral Delivery of tsTAG

RIN 1046-38 cells were infected with adeno/RIP-tsTAG at varying multiplicities of infection (MOI). The virus was left on the cells for 2 hours then washed off and the cells received fresh medium. The infected cultures were incubated at 37°C for 48 hours, and then were shifted to 33.5°C for an additional 48 to 72 hours. The cells were washed with PBS and then fixed in Carnoy's fixative for immunocytochemistry using an anti-TAG antibody (Santa Cruz Biotechnology) to detect TAG expression. Approximately 10 to 20% of the RIN cells were intensely stained for TAG expression at MOIs of approximately 30 to 300 viral particles per cell.

To provide a higher probability of obtaining β-cell lines, recombinant plasmids utilizing the insulin promoter engineered for enhanced activity are constructed (see Example 8). These constructs provide β-cell specific expression of the oncogene, and in the case of the insulin promoter with enhanced function, also provide a level of gene expression nearly equivalent to that achievable with the CMV promoter.

C. Adenovirus Infection of Rat Islet Primary Cultures

The media was aspirated off 6 well cluster dishes containing primary cultures of islets with the cells well attached to dishes. Then 2 ml of M199 media/10% FBS containing 1000 pfu/cell was added (estimating 500,000 cells from 500 islets and 5 × 10^8 pfu/2 ml media). The sample was incubated at 37°C for 1 hour, the media was aspirated, and then 6 ml of M199/4% FBS was added. The sample was cultured for 24 hours, and then expression was checked.

Representative cultures of dispersed and whole islet preparations on both HTB-9 matrix (2 day old cultures) and matrigel matrix (6 day old cultures) were infected with adenovirus expression vectors for β-galactosidase under control of either the CMV promoter (pAC-CMV-β-gal) or the rat insulin 2 gene promoter (pAC-RIP-β-gal). After 24 hours, cultures were cytochemically stained using the β-galactosidase substrate X-gal to characterize expression efficiency. Fresh stain containing 1.75 mM $K_3Fe(CN)_6$, 1.75 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg/ml X-gal in water was made up. The cells were washed once with PBS, and then fixed for 20 min at room temperature in 0.5% formaldehyde. The cells were washed again with PBS, 1 ml of stain was added, and the sample was incubated for 30 min at 37°C. The cells were then washed once with PBS. In all culture preparations, dispersed and whole islet on matrigel or HTB-9 matrix, staining appeared faster, more intensely, and with higher frequency (greater than 80% of cells) in cultures infected with pAC-CMV-β-gal than in cultures infected with pAC-RIP-β-gal (about 50% of cells).

These results indicate that CMV is a more efficient gene promoter in cultured rat islet cells than the rat insulin promoter, although at this time it cannot be ruled out that the difference in β-gal expression under these promoters was due to differences in the titer of viable adenovirus used to infect the islet cultures. It was also observed that fibroblasts stained for the presence of β-galactosidase in cultures infected with pAC-CMV-β-gal but did not stain in cultures infected with pAC-RIP-β-gal, indicating a specificity for RIP promoter expression in islet β-cells. These studies demonstrate the feasibility of maintaining primary cultures of islet tissue and using adenovirus expression systems to modify protein production of these cultures.

D. Expression in Neuroendocrine Cells Using the α-glycoprotein Promoter

The pituitary gland secretes a number of different hormones including leutenizing hormone (LH), thyroid stimulating hormone (TSH) and follicle stimulating hormone (FSH) using a regulated secretory pathway. Each of these hormones contain an alpha and beta subunit. The beta subunits are expressed only in the appropriate pituitary cell types, giving specificity to each hormone. The alpha subunit, called α-glycoprotein, is common to all pituitary hormones and is expressed in all pituitary cell types. Although expression of this protein is fairly ubiquitous in the pituitary, it is postulated to be specific to neuroendocrine cell types only. In transforming pituitary tissue and/or pituitary tumors, the α-glycoprotein promoter may aid in expression of transforming proteins within neuroendocrine cells only and not within non-neuroendocrine cell types which may be also be present in the culture or tumor.

The α-glycoprotein promoter (Genbank accession number LO5632) was amplified by PCR from human liver DNA (Clontech) using Taq Plus Long (StrataGene). Oligonucleotides AT255 (5' GGGGAACTAGTAAACTCTTTGTTGAAG 3'; SEQ ID NO:14) and AT256 (5' CTCAGTAACTCGAGTTAATGAAGTCCT 3'; SEQ ID NO:15) were used in 40 rounds of PCR with denaturation at 94°C (30 sec), annealing at 55°C (30 sec) and extension at 72°C (2 min) to amplify the promoter. The promoter was cloned as a *SpeI/XhoI* fragment into the *SpeI/SalI* site of BetaGene plasmid BL436 (CMV-neo), creating BetaGene plasmid DM102 (α–glycoprotein-neo).

AtT20, RIN38, and H03 cells were transfected with BetaGene plasmids BL436 (CMVneo), BY428 (RIPneo) and DM102 (α–glycoprotein-neo) by electroporation as described herein. Clones resistant to G418 were counted after 13 days of selection. Pituitary cells (AtT20) transfected with BY428 did not survive selection with G418. DM102 created about 75% fewer clones than BL436 in the same cell line. In RIN38 (rat insulinoma) and H03 (human neuroendocrine) cells, DM102 colony formation was equivalent to BY428 with BL436 creating 75% more clones. These data indicate that the α–glycoprotein promoter provides neuroendocrine-specific gene expression.

EXAMPLE 8

Modified Insulin Promoters

The rat insulin 1 gene promoter fragment (RIP) was modified in an attempt to strengthen its transcriptional activity. The principal modification involved the attachment of varying numbers of Far-FLAT minienhancers (FF minienhancer) (German *et al.* 1992a) to different positions within an intact RIP or to a RIP truncated at -205 (-205RIP). FF minienhancers were constructed by generating oligonucleotides corresponding to the region of RIP between -247 and -196: 5'-GATCCCTTCATCAGGCCATCTGGCCCCTTGTTAATAATC*G*A*C*T*G*ACCCTAGGTCTAA-3' (top strand; SEQ ID NO:5); and 5'-GATCTTAGACCTAGGGT*C*A*G*T*C*GATTATTAACAAGGGGCCAGATGGCCTGATGAAGG-3' (bottom strand; SEQ ID NO:6). The underlined sequences at the ends of the oligonucleotides are *Bam*HI and *Bgl*II recognition sites. The oligonucleotides were annealed and ligated in the presence of restriction enzymes *Bam*HI and *Bgl*II. Since *Bam*HI and *Bgl*II produce compatible DNA ends but can no longer be digested by *Bam*HI or *Bgl*II, the only multimers that escaped *Bam*HI and *Bgl*II digestion were ligated head-to-tail.

Minienhancers in which the three italicized bases in SEQ ID NO:5 and SEQ ID NO:6 above were mutated are called FFE minienhancers. FFE minienhancers are constructed essentially as described above by generating oligonucleotides corresponding to the region of RIP between -247 and -196: 5'-GATCCCTTCATCAGGCCATCTGGCCCCTTGTTAATAATC*T*A*A*T*T*ACCCTAGGTCTAA-3' (top strand; SEQ ID NO:7); and 5'-GATCTTAGACCTAGGGT*A*A*T*T*A*GATTATTAACAAGGGGCCAGATGGCCTGATGAAGG-3' (bottom strand; SEQ ID NO:8). The italicized bases represent the mutated bases. FFE minienhancers were shown to be more active than FF minienhancers when both are attached to a minimal promoter (German *et al.* 1992a). FF and FFE minienhancer dimers, trimers, *etc.* were separated by polyacrylamide gel electrophoresis and blunt-end cloned into the transient transfection vector, pBS/RIP/hGH, at either a *Xho*I site immediately upstream of -415 of the intact RIP, into an *Avr*II site at -206 of an intact RIP, or into an *Apa*I site immediately upstream of -205RIP. The number and orientation of minienhancer repeats were verified by DNA sequencing.

FF and FFE minienhancer/RIP-hGH constructs were transiently cotransfected along with Rous sarcoma virus-chloramphenicol acetyltransferase (RSV-CAT), an internal control plasmid used to monitor differences in transfection efficiencies, into 1×10^7 RIN cells by electroporation (Chu and Berg 1987) as modified by Bassel-Duby *et al.* (1992). The cells were incubated overnight in 199 medium containing 5 mM butyrate. The next day 199 medium containing butyrate was removed and new medium without butyrate was placed on the cells. After 48 to 96 hours, expression of the transfected genes was measured by hGH protein accumulation in the culture medium (Selden *et al.* 1986) using a radioimmunoassay (Nichols Institute, San Juan Capistrano, CA). The cells were harvested after these time points and extracts were prepared by three successive rounds of freezing and thawing. CAT activity in the cell extracts was determined by the method of Nielsen *et al.* (1989). Promoter activity as measured by hGH production was then normalized for transfection efficiency differences between samples by the quantitated CAT activity in each sample.

The activities of several FF and FFE/RIP promoters were compared to RIP activity in transiently transfected RIN cells. The best results were obtained with one of the types of FFE minienhancer-RIP constructs. This type of RIP derivative had either three or six copies of the 55 bp FFE minienhancer fused immediately upstream of -410 of intact RIP (pFFE3/-415RIP/hGH and pFFE6/-415RIP/hGH). These modRIP promoters were consistently 5- to 6-fold more active than unmodified RIP in RIN cells (FIG. 11). A number of other RIP derivatives were also more active than RIP in transient transfection assays, although not to the same extent as FFE3/-415RIP and FFE6/-415RIP.

The strength of the modified RIP promoters was also determined in stably transfected RIN cells. The stable transfection vector, pFFE3/RIP8/INS/IRES/NEO containing three copies of FFE minienhancers (FFE3), was generated by inserting a blunt-ended *Kpn*I/*Hin*dIII FFE3/RIP into pCMV8/INS/IRES/NEO in which the CMV promoter was removed with *Spe*I and *Sac*I. pFFE6/RIP8/INS/IRES/NEO was constructed by inserting an *Apa*I/blunt-ended

*Hin*dIII FFE6/RIP fragment into pRIP8/hGH polyA in which RIP was removed by *Apa*I/*Eco*RV. A *Bgl*II/*Stu*I INS/IRES/NEO fragment was then inserted into pFFE6/RIP8/hGH polyA to complete pFFE6/RIP8/INS/IRES/NEO.

In some of the stable transfection vectors, the normally used adenovirus-immunoglobulin hybrid intron was replaced with the rat insulin 1 gene intron (RIPi). RIPi was obtained by polymerase chain reaction from rat genomic DNA using oligonucleotides 5'-CTCCCAAGCTTAAGTGACCAGCTACAA-3' (SEQ ID NO:9) and 5'-GGGCAA CCTAGGTACTGGACCTTCTATC-3' (SEQ ID NO:10). These oligos produced a 185 bp product containing the 119 bp RIPi (Cordell *et al.* 1979) and a *Hin*dIII site on the 5' end and a *Bam*HI site on the 3' end. The PCR product was digested with *Hin*dIII and *Bam*HI and ligated into pNoTA/T7, whereupon it was removed with *Xba*I blunt-ended with Klenow/*Hin*dIII and inserted into *Eco*RV/*Hin*dIII digested pRIP8/INS/IRES/NEO to generate pRIP8/RIPi/INS/IRES/NEO. pFFE6/RIP8/RIPi/INS/IRES/NEO was constructed by replacing the 5' adenovirus-immunoglobulin hybrid intron/INS/IRES of pFFE6/RIP8/INS/IRES/NEO with RIPi/INS/IRES from pRIP8/RIPi/INS/IRES/NEO.

As with the transient transfection data, several modRIP promoters also appear to have increased activity compared to that obtained for RIP in stably transfected RIN cells. Both insulin mRNA and secreted insulin protein levels in stably transfected RIN cells were three to four times higher in FFE6 derivatives than levels obtained for RIP alone (FIG. 12A, FIG. 12B and FIG. 12C). In fact, the activity of FFE6 derivatives approached the level of activity exhibited by CMVp in stably transfected RIN cells.

FFE6 promoters also proved to be cell-specific. FFE6 promoters were fused with the neomycin gene to generate FFE6/RIP8/NEO. This plasmid was stably transfected into RIN cells, 293 cells, and pituitary AtT-20 cells. When challenged with G418, drug-resistant colonies were only present in RIN cells. As a control, CMV/NEO was also stably transfected into RIN cells, 293 cells, and pituitary AtT-20 cells. After selection in G418, a large number of drug-resistant colonies were present in all three lines.

Therefore, RIP derivatives like FFE6/RIP8/RIPi possess two important characteristics necessary for optimal expression of linked transforming genes in human β-cells: 1) they will direct expression of the transforming gene to β-cells and remain silent in other cell types associated with the islet preparation; and 2) they will deliver high levels of the transforming gene similar to those obtained from the very strong, non-cell-specific CMVp.

EXAMPLE 9

Induction of β-Cell Growth

A. Background

Although diabetes can be alleviated by insulin injections or sulfonylureas, the fine tuning of blood glucose sensing is lost which, more often than not, leads to unfortunate diabetic complications. Islet transplantation is a frequently mentioned alternative treatment for diabetes that retains glucose sensing, but this remains problematic, mostly because the quantity, quality and consistent supply of human isolated pancreatic islets suitable for transplantation is severely limited. One possible means of circumventing this problem is to somehow induce human β-cells to grow, either to increase the number of human islet β-cells or to produce a human β-cell line suitable for transplantation. However, little progress has been made to find a means that urges islet β-cells into a growth phase to such an extent where large quantities of β-cells can be produced as a potential alleviation of diabetes, and no human β-cell lines that retain essential traits required from *in vivo* insulin delivery have been created or isolated.

Pancreatic islet β-cell growth can occur from two separate pathways (Swenne, 1992). New islets can differentiate from budding of pancreatic ductule epithelium (neogenesis), or from the replication of existing islet β-cells. Neogenesis of islets is thought to primarily occur during fetal and perinatal stages of development, but has also been observed in the regenerating adult pancreas (Bonner-Weir, 1992). Replication of existing pancreatic β-cells has been seen in the late fetal stages, but is thought to be the principal means of increasing β-cell mass in the adult (Swenne, 1992). In a population of normal islet β-cells the number that are under going cell-division has been measured to be between 0.5-2%.

Several factors have been shown to increase the number of replicating β-cells, however these factors have had only slight effects. Glucose and other nutrients metabolized by the β-cell can increase the number of replicating adult β-cells 2-fold (Hellerström *et al.*, 1988). Several peptide growth factors have shown stimulation of β-cell replication (Bonner-Weir, 1992). Growth hormone (GH) increases the number of β-cells replicating in islets to around 6% (Nielsen *et al.*, 1989). The expression of the GH-receptor has been identified on β-cells (Hellerström *et al.*, 1991). The GH related peptides, prolactin and placental lactogen have similar stimulatory effects on β-cell replication reflecting lactogenic as well as GH-receptors on the β-cell surface (Moldrup and Nielsen, 1990).

It has been suggested that GH mediates its growth effect on β-cells by stimulating the production of IGF-I in islets which in turn mediates a paracrine or autocrine effect to stimulate β-cell replication (Nielsen, 1982). While this may in part be so, (indeed IGF-I alone has been shown to stimulate fetal β-cell replication 2-fold (Brelje and Sorenson, 1991)), it is also clear that GH can exert a stimulation of adult β-cell replication independently of IGF-I (Swenne *et al.*, 1987). Gastrin and cholecystokinin can instigate a small increase in β-cell replication (Bonner-Weir, 1992).

In contrast, EGF does not appear to affect β-cell replication even though significant EGF binding to β-cells has been observed (Nielsen, 1989), suggesting that the EGF signal transduction pathway is not functional in pancreatic β-cells. Similarly, PDGF does not appear to affect β-cell replication, but this is due to there being very few PDGF-receptors on β-cells. However, when the PDGF β receptor is transfected into β-cells only a 50% increase in DNA synthesis was observed upon stimulation with PDGF β-chain (Welsh *et al.*, 1990), suggesting that a post-receptor signal transduction mechanism for β-cell replication is only partly present.

Very little work has been done on establishing key elements in mitogenic signal transduction pathways in pancreatic β-cells. However, insulin promoter driven SV40 T-antigen overexpression in transgenic mice has significantly induced β-cell growth and dedifferentiation resulting in the generation of insulinoma cell lines (Efrat *et al.*, 1988;

Miyazaki *et al.*, 1990). In other cell types, the T-antigen mitogenic signaling pathway is thought to be mediated by inducing Shc tyrosine phosphorylation, recruitment of Grb2 and Ras activation *via* induction of SOS (the Ras guanine exchange factor; Dikworth *et al.*, 1994). This suggests that in pancreatic β-cells a mitogenic signal transduction pathway mediated *via* Ras activation can be induced. However, in normal islet β-cells proto-oncogene expression is undetectable or extremely low, but in islets transfected with v-src, or a combination of c-myc and c-Ha-ras, only a modest 50% increased cell replication rate was observed (Welsh *et al.*, 1987). Thus, these studies imply that it is important to appropriately activate a mitogenic signal transduction pathway in β-cells as well as to overexpress certain key elements within that pathway.

Several growth factors have been shown to modestly induce β-cell mitogenesis, but as of yet no *potent* stimulator(s) of β-cell growth has been identified. Furthermore, the necessary stimulator(s) for signal transduction pathways of growth factor stimulated β-cell mitogenesis is quite poorly defined. Indeed, a given growth factor stimulation of islet β-cells could actually be ineffective because certain elements of the signal transduction pathway are either not appropriately expressed and/or activated.

The present Example concerns the identification of mitogenic signal transduction pathways in pancreatic β-cells, which in turn indicates an appropriate growth factor and signaling pathway to exploit for inducing β-cell growth *in vitro* and/or establishing novel β-cell lines. The inventors have found that IGF-1 and activation of a signal transduction pathway *via* IRS-2 and p70^{S6K} can induce up to a 30-fold increase in β-cell growth in insulinoma cells. Recombinant adenoviruses were generated to overexpress the IGF-1 receptor and/or IRS-2 in primary isolated islets (preferably human islets) to determine the effects of IGF-1 induced β-cell growth.

B Methodological Approach

Recombinant Adenovirus Construction. In order to obtain high overexpression and efficient gene transfer of mitogenic signal transduction proteins in primary islets, the recombinant adenovirus system was used. Essentially, replication deficient recombinant adenoviruses were constructed (Becker *et al.*, 1994a; Becker *et al.* 1994b). Initially, adenoviral constructs to markedly increase *in vitro* islet β-cell expression of IRS-1 (as a putative control for IRS-2), IRS-2, IGF-1 receptor, and insulin receptor (as a putative control for IGF-1 receptor) were produced. Both human and rat forms of these genes (GenBank Accession number S62539 (Rat IRS-1), X58375 (Human IRS-1), AF087674, AF083418 and AF050159 (Rat IRS-2), AB000732 (Human IRS-2), M27293 (Rat IGF-1 receptor), X04434 (Human IGF-1 receptor), M29014 (Rat insulin receptor) and A18658 (Human insulin receptor)) were obtained for expression in both human and rat isolated islets. A series of constitutively on/off IRS-1 and -2 variants are also available.

Recombinant adenoviruses where IRS-1, IRS-2, IGF-1 receptor, or insulin receptor expression is driven by the ubiquitous CMV-promoter (using pAC-CMV) were generated, and confirmed by restriction enzyme and sequence analysis. Alternatively, recombinant adenoviruses for specific β-cell expression driven by the insulin promoter (using pAC-RIP) are generated. Recombinant adenoviruses expressing β-galactosidase and luciferase driven by the CMV-promoter are used as controls. For insulin promoter driven expression a pAC-RIP driven luciferase expressing recombinant adenovirus is used as a control. Recombinant adenovirus infection of isolated islets is performed (Becker *et al.*, 1994), and IRS-1, IRS-2, IGF-1 receptor and insulin receptor overexpression in islets is confirmed by Northern analysis and immunoblotting.

Recombination of the pAC and pJM17 vectors to generate E1A deficient recombinant adenovirus can only accommodate an ~3.8 kb insert into pAC shuttle vector. However, the IRS-1, IRS-2, IGF-1 receptor, and insulin receptor cDNA inserts are all >3.8 kb. Therefore, an E3 deficient vector (in this case pBHG11) instead of pJM17 is used to generate E1A and E3 deficient recombinant adenovirus. The pBHG11 vector enables inserts of up to 9 kb into pAC to be used which is suitable for IRS-1, IRS-2, IGF-1 receptor, and insulin receptor cDNAs.

Cell Preparations. Isolated rat islets are used as a primary model, which are isolated as previously described (Alarcón *et al.*, 1993). The studies are then repeated in human islets.

Further characterization of mitogenic signal transduction pathways in the RITz and the INS-1 cell lines is conducted, and these cells are used as positive controls for investigation of IRS-2/IGF-1 receptor overexpression in islets. RITz-cells are isolated from the well granulated line of NEDH-rat transplantable insulinoma tissue by cellular sieving and Percoll™ centrifugation gradient purification. They are maintained in culture under identical conditions for INS-1 cells (Alarcón *et al.*, 1993). In terms of insulin secretion, RITz-cells are not responsive to glucose in the physiological range, but are when elevating intracellular cAMP, phorbol esters, and/or depolarization.

The occurrence of increased IRS-2 expression in human insulinoma tissue when compared with human islets is also confirmed. Human insulinoma tissue (for example, obtained from the Mayo Clinic) is used for this purpose.

Measurement of Cell Growth. Several parameters are used for measurement of cell growth. First, the cell number is counted in a standard volume using a heamocytometer. ^{3}H-thymidine incorporation into cellular DNA is used as a predictor of β-cell growth (Myers and White, 1996). Following addition of [^{3}H]-thymidine (1 μCi/ml) to cells, incubations were performed under various conditions for 2 –4 hr at 37°C. Cells were then washed three times in ice-cold HBBSS, lysed in 2 ml of 1 mg/ml SDS solution on ice, and transferred to 12-ml tubes. Following addition of 2.5 ml ice cold 20% (wt/vol) trichloroacetic acid, the cell extract was poured over a Whatman glass-fiber filter in a Millipore filtration apparatus. The filter was washed twice with ice-cold 10% (wt/vol) trichloroacetic acid, air dried, and counted by liquid scintillation counting. The individual replicating β-cells in islets or β-cell lines are identified and counted using a BrdU-staining kit (Amersham). This technique has the advantage of readily distinguishing between islet β-cells and non β-cells by double staining with a second antibody against insulin. An increase in a population of β-cells could result in part from an inhibition of β-cells entering apoptosis. Thus the number of apoptotic IRS-2/IGF-1 receptor overexpressing cells is also measured by the TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) method (kit obtained from Upstate Biotechnology Inc.).

Measurement of β-*cell Differentiation.* Ideally, when inducing primary islet β-cells to grow, the maintenance of as much of the β-cell's differentiation state as possible is desired. However, when either the growth rate of β-cells is increased or β-cells are transformed (*e.g.*, by X-ray exposure (RIN-cell lines) or β-cell specific T-antigen expression (βTC3- and MIN6 cells) there appears to be some degree of loss of differentiation (*e.g.*, glucose-regulated insulin release and biosynthesis). Thus, the differentiation state of primary rat and/or human islets induced to grow by in IRS-2/IGF-1 receptor overexpression is determined.

The (de)differentiation state is assessed using three parameters: *1) Glucose-regulated proinsulin biosynthesis translation-* To date all the available transformed β-cell lines (except the relatively well differentiated low passage MIN6 cells) do not possess a phenotype of specific regulated proinsulin biosynthesis by a physiologically relevant range of glucose concentrations. Maintenance of correct glucose stimulated proinsulin biosynthesis in IRS-2/IGF-1 receptor overexpressing islet β-cells (Alarcón *et al.*, 1993) is an indication of maintaining a differentiation state. *2) Regulated (pro)insulin release-* Dedifferentiated transformed β-cell lines have a tendency to constitutively secrete an increased proportion of proinsulin, and also lose their response to relevant secretagogues, especially glucose in the 2-20 mM range. Pulse-chase radiolabeling protocols (Alarcón *et al.*, 1995) are used to assess the proinsulin:insulin ratio released from IRS-2/IGF-1 receptor overexpressing islet β-cells in response to glucose and a stimulatory cocktail containing multiple secretagogues and potentiators of glucose-stimulated insulin secretion and thus assess the differentiation state. *3) $p70^{S6K}$ phosphorylation state-* The extent of $p70^{S6K}$ phosphorylation in β-cell lines tends to correlate with dedifferentiation state and growth rate of the cells. A rank order of serum-starved cultured β-cells is seen from differentiation (slow growing) to dedifferentiation state (rapid growth rate) of primary islets > MIN6 > INS-1 > RIT*z* > other RIN lines = βTC3 cells. The phosphosphorylation of $p70^{S6K}$ occurs on multiple sites on the molecule, with 5 phosphorylated forms observed by immunobloting (due to electrophoretic retardation of the $p70^{S6K}$ phospho-forms on SDS-PAGE); the upper 3-5 multiple phosphorylated $p70^{S6K}$ forms are activated. In islets only the non-phosphorylated form of $p70^{S6K}$ is observed, but in βTC3 cells only the fully phosphorylated active $p70^{S6K}$ is observed. Other β-cell lines rank in between these extremes. Thus immunoblot measurement of p70^{S6K} phosphorylation state is a rapid and convenient indication of β-cell differentiation state.

Characterization of β-*cell Mitogenic Signal Transduction Pathways-* Induction of β-cell growth/transformation requires not only overexpression of a particular element in a mitogenic signal transduction pathway (*i.e.*, IRS-2), but also activation of that pathway by an appropriate growth factor (*i.e.*, IGF-1). Therefore, in IRS-2/IGF-1 receptor overexpressing islet cells it is important to assess activation of the IGF-1 signal transduction pathway(s). This is performed using established methods (Myers and White, 1996). Changes in protein-protein interactions is measured (*e.g.*, IGF-1 instigated IRS-2-PI3 kinase association by immunoprecipitation with p85 PI3 kinase antibody followed by immunoblotting with either anti-phosphotyrosine and/or IRS-2 antibodies); the phosphorylation state of a particular protein is determined (*e.g.*, using specific antibodies that recognize only phosphorylated MAP kinase, or immunoblotting for gel retardation analysis of p70^{S6K} or immunoprecipitation followed by anti-phosphotyrosine immunoblotting); and induction of enzyme activity is measured (*e.g.*, MAP kinase or PI3 kinase assays). Necessary reagents or assay kits are purchased form UBI.

C. Results

To screen for factors that might be important in mitogenic signal transduction pathways in pancreatic β-cells, the expression of certain genes in a model of rapidly growing β-cells (*i.e.*, NEDH-rat transplantable insulinoma cells (Chick *et al.*, 1977)) versus a model of slow growing β-cells (*i.e.*, NEDH-rat normal isolated islet cells) was compared by Northern blot hybridization. Preproinsulin mRNA levels drop by 75% in insulinoma cells compared to islets. Levels of mRNA for c-jun, c-fos, and IRS-1 did not change when comparing islets to insulinoma cells. In contrast to IRS-1, mRNA levels for IRS-2 were increased >50-fold in insulinoma cells compared to isolated islets. These very high levels of IRS-2 mRNA were also found in RIN 1046-38, RIN-m5F, INS-1, βTC3, HIT and MIN6 cell lines, but not in βTC-1, AtT20, PC-12, GH-3, 293, Cos, CHO or 3T3-L1 cell-lines where IRS-2 mRNA levels were comparable to those in isolated rat islets.

The elevated IRS-2 levels appear to be peculiar to insulinoma cell lines. The only other gene product found so far to be overexpressed to such an extent in insulinoma cells is a Ha-Ras containing VL30 transposon element (*i.e.* an endogenous retroviral like transposon that contains the Ha-Ras sequence within it). However, the overexpressed VL30 element mRNA is not reflected in Ha-Ras expression at the protein level which is unchanged compared to normal rat islets. Thus, this particular VL30 is acting like a typical transposon that is quite common to tumor cells. The elevated levels of IRS-2 mRNA in insulinoma cells were also reflected at the protein level by immunoblot analysis. Furthermore, the levels of other potential mitogenic signal transduction proteins in the β-cell, namely IRS-1, c-Ha-Ras, PI3-kinase, $p70^{S6K}$, Shc, Grb-2, MAP-kinase (erk-1 and -2 isoforms) and CREB were not changed between islets and insulinoma cells.

A polyclonal cell line from the NEDH-rat transplantable insulinoma tissue termed RITz-cells has been obtained. When starved of serum for 48h RITz-cells continue to grow, albeit at a slower rate, so that the rate of ^{3}H-thymidine incorporation drops 4-fold compared to fed cells. However, upon refeeding RITz-cells with 10% (v/v) serum the ^{3}H-thymidine incorporation rate increases by 20-fold after a further 48h incubation, in line with a parallel increase in RITz-cell proliferation. Interestingly, the expression levels of the aforementioned signaling molecules (including the IRS-2 overexpression) did not significantly change in these ± serum studies. In addition, the differentiation state (as judged by secreted proinsulin:insulin ratio and regulated insulin secretory response to a stimulatory cocktail (20 mM glucose, 10 μM forskolin, 1 mM IBMX, 30 mM KCl, 50 μM PMA) did not alter in the same ± serum studies.

The question as to which growth factor(s) in the serum is responsible for this marked stimulation of β-cell growth was also addressed. IGF-1 at a concentration of 10^{-9} M was found to give a maximum stimulation (>30-fold; ED_{50} ~10^{-10}M IGF-1) of RITz-cell growth (as analyzed by [^{3}H]-thymidine incorporation) after a period of 48h serum deprivation. There is no additive or synergism of serum (10% v/v) + IGF-1 (at 10^{-9} M), suggesting that it is IGF-1 in serum that is responsible of stimulating RITz-cell growth. Interestingly, unlike the majority of other cells in tissue culture, the RITz-cells (and also INS-1 and βTC3 cells) do remarkably well in the absence of serum for periods up to 5 days, although they do grow at a slower rate. It is possible that insulin secreted by such insulinoma cells is 'feeding back' *via* the IGF-1 receptor to maintain the cell line.

Preliminary characterization of the mitogenic signal transduction pathway stimulated by IGF-1/serum in RITz-cells was conducted. Identical observations are obtained whether IGF-1 and/or serum is used as a stimulation, but only IGF-1 stimulation will be referred to below. Addition of IGF-1 (10^{-9} M) to 48 h serum-starved RITz-cells followed by a 1 h incubation induced autophosphorylation of the IGF-1 receptor, gave a marked stimulation of tyrosine phosphorylation of IRS-2 and an increased association of PI3 kinase (PI3K; 85 kDa subunit) which in turn activates PI3-kinase activity. This then increased the phosphorylation state of $p70^{S6K}$, and hence its activation. Activation of $p70^{S6K}$ has been implicated in mitogenic stimulation in other cell types (Myers and White, 1996). In contrast, no increased tyrosine phosphorylation of Shc or phosphorylation activation of MAPK activity was found.

Next the serum effects on activation of the IGF-1 signal transduction pathway in INS-1 cells was investigated. IRS-2 is activated by tyrosine phosphorylation within the 10-30 min window, resulting in increased association of PI3'K to IRS-2 in INS-1 cells, as shown by immunoprecipitation with a PI3'-kinase 85 kDa subunit antibody, and subsequent antiphospho-tyrosine and/or IRS2 immunoblotting analyses in 48 h serum-starved INS-1 cells that have been re-fed with serum for 10 min or 30 min. Similar results are observed with IGF-1 stimulation. In INS-1 cells serum starved for 0-72 h, refeeding with 10% (v/v) serum induces an increased activation of $p70^{S6K}$ by increasing its phosphorylation state, as indicated by a retarded mobility on gel electrophoresis.

Similar evidence has been obtained for activation of $p70^{S6K}$ by IGF-1. Several bands of $p70^{S6K}$ can be observed, which correlate to differentially phosphorylated isoforms of the enzyme. In 48h serum starved cells MAPK is endogenously activated, using immunoblotting studies comparing specific antisera that only recognizes the phospho-activated form of MAPK versus antisera which recognizes total MAPK whether phosphorylated or not. However, if INS1 cells are both serum and glucose starved for 48 h, activation of MAPK within 10 min exposure to 15 mM glucose alone can be observed. Conversely, also in INS1 cells both serum and glucose starved for 48 h, adding back 10% serum for 10 min results in activation of MAPK by 10% serum. Thus, both glucose and serum (*i.e.* IGF-1) can activate MAPK in β-cells. Similar effects of IGF-1 are also being observed to that of adding back 10% serum. Characterization of IGF-1 activation of signal transduction pathways in the β-cell is being studied to identify other elements of this cascade that result in β-cells growth.

The data suggest that the IGF-1 signal transduction pathway in β-cells occurs preferably *via* a IRS-2/p70^{S6K} route, rather than a route involving activation of Ras. Because of the massive overexpression of IRS-2 in insulinoma cells, it appears that IGF-1 signaling is mediated *via* IRS-2 rather than IRS-1. As previously stated, IRS-2 expression levels did not change in response to adding back serum and/or IGF-1. This latter observation suggests that it is not only IRS-2 overexpression, but also activation of IRS-2/p70^{S6K} signal transduction pathway that is important for IGF-1 mediated stimulation of β-cell growth. This indicates that IRS-2 (and possibly IGF-1 receptor) overexpression in primary (human) islets initiates an IGF-1 mediated potent stimulation of β-cell mitogenesis and/or leads to a novel (human) β-cell line (FIG. 13).

A background level of glucose is required for IGF-1 to stimulate mitogenesis of INS-1 cells (as judged by [^{3}H]-thymidine incorporation). In considering that INS-1 cells respond to glucose in terms of insulin secretion in the appropriate physiological range, for any significant IGF-1 stimulation of INS-1 cell growth glucose must be present > 3 mM glucose (FIG. 14). At 10 nM to 3 mM glucose IGF-1 only has a slight effect in stimulating INS1 cell growth. Glucose alone can instigate INS1 cell growth in a dose dependent manner ~3-fold at 6 mM glucose, ~4-fold at 9 mM glucose and ~10-fold at 18 mM glucose. This effect of glucose on INS1 cell growth is potentiated by IGF1 in a dose dependent manner at >10 pM IGF-1 reaching a maximum between 10-100 nM IGF-1. The role that glucose plays in IGF-1 mitogenic signaling pathways in pancreatic β-cells is investigated by studying phosphorylation activation of the 'signal transduction proteins' and protein-protein interactions by IGF-1 ± glucose. It is known that glucose is capable of activating MAPK (*via* a Ca^{2+}-

dependent process), therefore its role in activation of other elements in that pathway is investigated.

Like IGF-1, growth hormone (GH) can also stimulate mitogenesis in β cells. However, it does not signal mitogenesis via the IRS-1/2 pathway, but via the JAK/STAT pathway. In particular, JAK2 and STAT5 A and B are involved in the mitogenic pathway (FIG. 13). The action of rat growth hormone (rGH) on INS1 cell growth, like that of IGF-1, requires a 'background' of glucose (FIG. 15). The rGH has no effect on INS-1 cell growth until a threshold of 6 mM glucose that reaches a maximum (~50-fold increase compared to "0" glucose) at 15 mM glucose. This is similar to the effect of IGF-1 on INS1 cell growth which has a threshold between 2-4 mM glucose and reaches a maximum at 15 mM glucose. Additionally, there is an additive effect of rGH and IGF-1 on INS-1 cell growth at glucose concentrations up to a maximum of 12 mM (FIG. 16).

In line with the observed effect of IGF-1, glucose and rGH stimulation of INS-1 cell growth, inhibitors of the mitogenic signal transduction pathway were shown to inhibit INS-1 cell growth. Rapamycin (a $p70^{S6K}$ inhibitor), wortmannin and LY294002 (PI3K inhibitors), and PD29083 (a putative MEK inhibitor) all inhibit IGF-1, rGH and glucose induced INS1 cell growth.

Adenoviral-mediated overexpression of IRS-2 in INS1 cells in the presence of IGF-1 and 15 mM glucose resulted in an approximately 200-fold increase in [^{3}H]-thymidine incorporation compared to uninfected INS1 cells plus no glucose (FIG. 17). The mitogenic signal was again specific for IRS-2 and not IRS-1 as INS1 cells infected with adenovirus-IRS-1 showed no mitogenic response over and above that for uninfected cells or cells infected with adenovirus-β–Galactosidase. As before, a background level of glucose was required to stimulate mitogenesis in the adenovirus infected cells. Interestingly, the mitogenic response of INS1 cells to adenoviral overexpression of IRS-2 was greater than that for large T-antigen, a protein known for its ability to induce dedifferentiation and subsequent mitogenesis (FIG. 17).

Overexpression of an element in the signal transduction pathway downstream of IRS-2 (*e.g.*, 'constitutively on' variant forms of p70^{S6K} and/or Ras (RasQ81L)) in islet β-cells is also contemplated. IRS-2 is a multiple tyrosine phosphorylated molecule that appears to be located at a crossroads for many mitogenic signal transduction pathways in a cell (Myers and White, 1996). One particular growth factor induces phosphorylation of only certain IRS-2 tyrosine residues, and thus limits the number of downstream elements that associate with IRS-2 and can then be activated. Furthermore, IRS-2 activation requires exogenous growth factor stimulation (even in IRS-2 overexpressing cells), thus activation of mitogenic signal transduction pathways *via* IRS-2 can be turned on and off (unlike overexpression of 'constitutively on' downstream elements). Thus continuing characterization of mitogenic signal transduction pathways in β-cells is being investigated to identify other candidates that induce β-cell growth.

EXAMPLE 10

BetaGene Medium Maintains Growth and Function of Neuroendocrine Cells

The biologic activity of peptides considered for biopharmaceutical applications are influenced by a number of complex modifications. These posttranslational modifications include correct proteolytic processing of precursor molecules, amidation, glycosylation, disulfide formation, folding, and oligerimization. Production in mammalian cell systems is necessary for many therapeutically relevant peptides to ensure bioactivity and minimize immunogenicity. The latter issue of immunogenicity may even require the use of human cell systems. Neuroendocrine cells are cells that are specialized in the biosynthesis and export (secretion) of biologically relevant peptides. A distinguishing characteristic of neuroendocrine cells is the dominance of a regulated secretory pathway. This pathway involves sorting to and storage of peptides in dense-core or secretory vesicles, in addition to both relatively high level biosynthesis and posttranslational modifications of peptides. In certain applications, neuroendocrine cells are used as a cellular therapy for *in vivo* delivery of bioactive peptides. Such applications require large-scale production of the implantable cells.

A number of enzymes that are essential for the posttranslational modifications have been characterized, with many abundantly expressed in neuroendocrine cells. Whether manufacturing processes utilizing neuroendocrine cells involve production of purified peptides or cells for implantation, the process must sustain the activity of these enzymes so that bioactive peptides will be produced. The present disclosure provides optimized culture media for neuroendocrine cells, for the purpose of not only growth, but also function, specifically, secretory function, and the functional activity of enzymes requisite for posttranslational processing. This has involved the use of primary human neuroendocrine cells and neuroendocrine cell lines (some specifically engineered to express therapeutically relevant peptides) to empirically determine components critical to secretion and processing.

A. Assay of Cell Growth- Neutral Red Uptake Assay

A neutral red uptake assay was used for quantification of viable cell mass to allow rapid determinations of cell growth, and for calculation of cell doubling times. Neutral red diffuses across cell membranes, while protonated neutral red does not. Neutral red accumulation is dependent on an acidic compartment (maintained by H^+/ATPase). Accumulation is time and concentration dependent, and with conditions appropriate to cells of interest, uptake is linearly related to viable cell number. The assay is initiated by adding neutral red (from 1 mg/ml stock in acetic acid) to cells to provide a final concentration of 25-50 μg/ml (a minimum of 2 ml medium/cm^2 culture surface in each well is required). The cells are then incubated with neutral red for 0.5-1 h at 37°C. The medium with neutral red is then aspirated, the cells washed once with medium and the neutral red is extracted from the cells. Neutral red is extracted with a solution containing 50% ethanol and 0.1 M NaH_2PO_4 (pH 5.1-5.5). The soluble neutral red is quantified by determining absorbance at 540 nm in a plate reading spectrophotometer, with a standard curve of neutral red (1-40 μg/ml) dissolved in the same extraction solution.

B. Assay of Peptides

Insulin in the medium was quantified with a commercial radioimmunoassay (DPC, Los Angeles CA), that is referenced to USP human insulin (lot G; US Pharmacopeia, Rockville

MD). The reference USP human insulin that is included in each assay is validated by HPLC as disclosed herein (Example 5).

Human growth hormone is determined with a human growth hormone ELISA (Boehringer Mannheim, Indianapolis, IN). The growth hormone (GH) standard was validated by western blotting and HPLC referenced to an independent source of human growth hormone (Bachem, Torrance CA).

C. **Cell Culture**

Four different neuroendocrine cell lines were used to evaluate the impact of the current medium on growth. Two cell lines are human; βG 785/5, an engineered version (these cells have been engineered to express human growth hormone) of a neuroendocrine line derived from a lung tumor (βG H04; ATCC CRL-5803) and a neuroendocrine gastric carcinoma (βG H16; ATCC CRL-5974). The other 2 lines are rodent cells, derived from a rat insulinoma, one engineered to express human insulin (βG 18/3E1; Clark *et al.*, 1997), the other, βG 191/26, engineered to express preproglucagon (transfected with BetaGene plasmid BU503; WO97/26334 and WO97/26321).

βG H16 cells were passaged with a 1:3 split ratio into 12 well plates and fed 2-3 times/week with 4 ml per well of BetaGene Medium supplemented with 2% or 5% serum or serum-free. βG 785/5 cells were plated (1:30) into 24 well plates, about 1 × 10^4/well, and fed 2-3 times/week with 2-3 ml per well of either RPMI or BetaGene Medium supplemented with FBS or serum-free. Media samples were collected for human GH assay, and cell growth determined at 2-3 day intervals for about 2 weeks. RPMI is the medium recommended for this cell type. βG 18/3E1 cells were plated (1:8 to 1:16) into 24 well plates, about 1 × 10^5/well, and fed 2-3 times/week with 2-3 ml per well of BetaGene Medium supplemented with 0.5-5% FBS or serum-free. Media samples were collected for human insulin assay, and cell growth determined at 2-3 day intervals for approximately 2 weeks.

D. Medium Supplements- Serum and Serum-free

Serum-supplemented media contained fetal bovine serum (JRH Biosciences, Lenexa KS), supplemented to 2%, unless otherwise indicated. The lot of serum used was selected by screening at least 5 lots of serum by assaying attachment, clonal growth, and maintenance of secretory function (of primary pancreatic β-cells and β-cell lines) at serum supplements of 0.5% to 5%. Serum-free supplement provided 0.1% BSA, 10 µg/ml of transferrin, and 50 µM each of ethanolamine and phosphoethanolamine.

E. Medium

The performance of cells in BetaGene medium (JRH Biosciences) was compared to RPMI, a medium recommended for culture of human cells (Ham and McKeehan, 1979; Acton *et al.*, 1979), and the medium recommended for the βG H04 cell line (the parental cell line engineered to yield the βG 785/5 cell line). βG H16 cells were derived and cultured in DMEM:F12 (50:50) mixture supplemented with a complex mixture of hormones, growth factors, selenium, BSA, transferrin, ethanolamine and phosphoethanolamine (10 µM each). For the present studies the βG H16 cells were switched to BetaGene Medium with either FBS or serum-free supplements and growth was evaluated in this medium.

F. Growth and Function

The βG H16 cell line is a slow-growing suspension culture with a 5-6 day doubling time. The βG 785/5 cell line is a rapidly growing monolayer culture that readily reaches confluence with a 2 day doubling time. The βG 18/3E1 cell line is a slower-growing monolayer culture that does not readily achieve confluence. Growth in BetaGene Medium for all these cell lines was maintained when serum-free supplements (SF) were used in the place of FBS (Table 10 below).

TABLE 10

Cells	Supplement	Doubling, days
H16	SF	5.91±0.41
	2% FBS	5.03±0.14
	5% FBS	5.31±0.23
βG 785/5	SF	1.97±0.01
	2% FBS	2.03±0.01
βG 18/3E1	SF	2.41±0.01
	0.5% FBS	3.85±0.14
	1% FBS	2.92±0.03
	2% FBS	2.83±0.02
	5% FBS	2.77±0.01

The βG 785/5 cell line was derived from βG H04 cells which were derived and routinely cultured in RPMI with FBS. The growth rate of βG 785/5 cells in BetaGene and RPMI media, with FBS or SF, is shown in FIG. 18. Although cells grown in RPMI with FBS exhibited a longer lag phase, the growth of cells in BetaGene medium and RPMI with FBS was similar, all with doubling times of 2 days. However, cells in RPMI with SF essentially failed to grow, with an apparent doubling time of 26 ± 1 days. Three wells of RPMI with SF were switched to BetaGene Medium with SF for the last 4 days of the experiment, resulting in a restart of growth and a doubling time of 3.2 ± 0.2 days.

In contrast with growth, the secretory function of cells in RPMI medium did not keep pace with cells grown in BetaGene Medium (FIG. 19, βG 785/5). The human growth hormone (hGH) output of cells grown in BetaGene Medium with FBS was approximately 5 times greater than growth hormone output from cells in RPMI with FBS. Similarly, the hGH output of BetaGene Medium with SF was more than 5 times that of RPMI with SF. While BetaGene Medium supplemented with SF sustained hGH output equal to that of RPMI with FBS, it was not sufficient to support the same secretory function as BetaGene Medium with FBS.

The growth of βG 18/3E1 cells was slowed only with low serum — at 0.5%, but not by SF-supplementation (Table 10 above). The insulin secretory function of these cells was maintained with all supplements until the cells reached the plateau phase of growth. Cells at plateau phase, whether supplemented with 0.5% FBS or SF, do not maintain normal secretory output (FIG. 20). This was confirmed in separate studies with SF and 0.5% FBS cultures. The secretory impairment at plateau phase under these conditions may be due to decreased biosynthesis or processing of insulin rather than an impairment of secretion. The ability to respond to a secretagogue cocktail is shown in FIG. 21 for SF- and 2% FBS- supplemented cultures in BetaGene Medium. This demonstrates that the capability of the regulated secretory pathway has been maintained, only the absolute output has been affected in both unstimulated and stimulated states, while the fold response is maintained. RPMI medium is one of the most commonly used media for culture of rat and hamster β-cell lines. The present results with BetaGene medium stand in contrast with the literature where insulinoma cells cultured in RPMI medium extinguish insulin production at plateau phase of cell growth (Karlsen *et al.*, 1991). Only with BetaGene medium supplemented with serum-free supplements is insulin output reduced at plateau phase of growth, although it is not extinguished.

The capability of BetaGene medium to sustain processing and secretion of a peptide that yields proteolytically cleaved and amidated products was evaluated by measuring GLP-1 (amidated and non-amidated) production. βG 191/26 cells were plated in T25 flasks with BetaGene Medium, and then the medium was switched to RPMI, RPMI with 75 μM ascorbate, or BetaGene Medium, all with 2% FBS. Both the total GLP-1 and the amidated GLP-1 output/day of cells in BetaGene Medium was essentially double that of cells in RPMI. The addition of ascorbate (in the form of the stable ascorbate-2-phosphate) to RPMI increased the amidated GLP-1 output to that of BetaGene Medium, but did not normalize the total GLP-1 output/day (FIG. 22).

EXAMPLE 11

Effects of Ascorbate 2 – Phosphate and Copper on Post-translational Modification

Amidation of a carboxy-terminal glycine is one of the later events in post-translational processing. This modification is essential for the activity of some peptides, including about half of peptide hormones, and appears to be rate-limiting for production of some peptides (Eipper *et al.*, 1992; Cuttitta, 1993). The bifunctional enzyme responsible for amidation is petidylglycine α-amidating monooxygenase (PAM). The enzyme itself is proteolytically processed and is both N- and O- link glycosylated and is targeted to secretory granules in neuroendocrine cells (Yun *et al.*, 1994). This enzyme requires copper and ascorbate to accomplish amidation; copper is a part of the functional enzyme.

Although several media include ascorbate in the formulation, the value of ascorbate has been most typically considered in the context of extracellular matrix and collagen synthesis. Even for the purpose of collagen formation the addition of ascorbate has been considered impractical in light of the instability of ascorbate (Ham and McKeehan, 1979; Mather, 1998). There are several analogues that are stabilized forms of ascorbate. One of these compounds is ascorbate-2-phosphate (A-2-P; Nomura *et al.*, 1969). This compound is used in some pet foods, and as a supplement in some types of cell cultures. A-2-P has been shown to stimulate collagen synthesis in fibroblast cultures. It has been used for culture of rat hepatocytes, although rat (unlike human) hepatocytes synthesize ascorbate.

Recently, A-2-P was shown to acutely improve glucose-induced insulin secretion from pancreatic islets of scorbutic guinea pigs (Wells *et al.*, 1995). These authors indicated that normal islets have intracellular levels of 5 μM, with scorbutic levels less than 10% of normal. In contrast, ascorbic acid has been reported to be acutely inhibitory in electrophysiologic and secretory studies with rodent β-cells (Bergsten *et al.*, 1994); these authors indicate that intracellular ascorbate concentrations in normal mouse islets are 4 mM. Ascorbic acid has been shown to be diabetogenic *in vivo*, toxic to mouse islets *in vitro* and to cultured fibroblasts, hepatocytes and lung carcinoma cells (Anderson and Grankvist, 1995). It is not clear what concentrations of ascorbate would be required by islets, whether ascorbate would be toxic with chronic culture, or whether there may be species differences in the effect of ascorbate in β-cells.

Ascorbic acid or a substitute reducing agent is utilized on an essentially equimolar basis for each mole of amidated product. The provision of ascorbate would then be expected to be important for maintaining peptide amidation with neuroendocrine cells cultured, particularly in the absence of serum, or grown at high-density production scale. One study of neuroendocrine cells engineered to express an amidated peptide (pancreatic polypeptide) was unable to increase amidation activity by supplementing with 50 mM ascorbic acid (Takeuchi *et al.*, 1991); maximal production achieved was approximately 6 pmol/million cells/day.

The present studies have used cultured primary human islets, rat β-cell lines, and human neuroendocrine cells to determine the chronic cytotoxic effects of ascorbate and A-2-P, whether A-2-P will support PAM-amidation activity, and whether A-2-P has any effect on the secretion of non-amidated peptides, such as insulin.

A. Instability of Ascorbate and Stability of A-2-P in Media at 37°C

The first consideration was to determine whether A-2-P was a more stable form of vitamin C in the cell culture environment. To that end an assay was devised that takes advantage of the dye reducing properties of ascorbic acid. The assay can be coupled with alkaline phosphatase to dephosphorylate A-2-P so that it can be measured with the same assay system used to measure ascorbate. The assay uses alkaline Tris-Mg buffer (pH 7.8-8.0; 2 mM $MgCl_2$) and nitroblue tetrazolium for ascorbate alone, or for A-2-P the solution contains in addition 10 U/ml of calf intestinal phophatase (C-AlkP). Ascorbate reduces the pale yellow NBT resulting in an intense purple color development. The color is developed whether the source is sodium ascorbate or ascorbic acid produced by the dephosphorylation of A-2-P by C-AlkP.

To prepare the assay solution, nitroblue tetrazolium was dissolved in 70% dimethylformamide to provide a 61 mM dye stock solution. Sodium ascorbate stock solution and A-2-P stock solution were made at 100 mM in RO/DI water or culture medium; ascorbic acid stock is stored frozen at less than -120°C. These stock solutions are used to construct a standard curve with a range of 1 to 18 mM in culture medium. The assay reaction mixture consists of 0.1 M Tris buffer, 1 mM magnesium, 0.4 µM nitroblue tetrazolium, with or without 10 U/ml of C-AlkP. The standards and samples, 10 µl, are pipetted into individual wells of a 96 well plate. The reaction is started by adding 100 µl of reaction mixture to each well. The reaction is quantified as a rate assay, with kinetic reading of OD at 595 nM at 20 sec intervals for 15 minutes. The stability of ascorbate was determined by spiking medium samples with ascorbic acid or A-2-P, then incubating the samples in the dark at 4°C, room temperature, and 37°C. The change in concentration with ascorbate and A-2-P, after 1 and 2 days at the various temperatures, is presented in Table 11 below. The results indicate that ascorbate in media is degraded quickly, with marked breakdown occurring at 4°C. In contrast, A-2-P was very stable with little loss of activity (98% recovery) after 4 days at 37°C. Refrigerated media exhibited the same A-2-P concentrations as freshly manufactured medium for times of > 6 months.

TABLE 11

Temperature, °C	Ascorbic Acid, mM		Ascorbate Phosphate, mM	
	1 day	2 day	1 day	2 day
-15	8.9 (99%)	6.8 (75%)	9.2 (102%)	9.6 (107%)
4	7.0 (59%)	5.4 (59%)	9.2 (102%)	9.4 (105%)
37	3.4 (38%)	2.4 (27%)	9.6 (107%)	11.4 (126%)

B. Toxic Concentrations of Ascorbate with Human Neuroendocrine Cells

βG H03, a lung neuroendocrine cell line, was engineered to express human insulin by transfection with BetaGene plasmid AA603. The resultant cell line, βG 498/45, biosynthesizes, processes, and secretes human insulin. A suspension culture of βG 498/45 cells (PD33) was plated in varying concentrations of ascorbate or A-2-P. Samples were collected for insulin assay and medium changed after 2 and 5 days of culture. In the initial 2 days of culture ascorbate altered insulin output by reducing insulin about 20% only at the highest concentration. In the final 3 days cells were dying and insulin output was reduced to about 20% of controls by the highest concentrations of ascorbate, while about 400 µM concentrations of both ascorbate and A-2-P enhanced insulin secretion (FIG. 23). The highest concentration of A-2-P did not inhibit insulin output.

C. Optimal A-2-P Concentration for PAM-activity of a Rodent β-cell Line

This activity assay used the βG 191/26 cell line engineered to overexpress the preproglucagon gene. Production of amidated and nonamidated GLP-1 was determined by immunoassay of secreted cell products from cells cultured 1 day in RPMI medium (with 2% FBS) supplemented with varying concentrations of A-2-P. The dose-response shows half-maximal and maximal amidation activity with about 1 and 10-100 μM of A-2-P (FIG. 24). The amount of amidated GLP-1 plateaued from 25-1000 μM. Concentrations of 10 mM consistently (4 separate experiments) resulted in slight decreases in amidated GLP-1, with a similar tendency to reduce non-amidated GLP-1 output. Supplementation with A-2-P results in a decrease in non-amidated GLP-1, such that amidated/non-amidated exceeds 100%. Maximal output of amidated GLP-1 with this cell line is about 12 pmol/million cells/day, representing a 5-fold increase over 0 μM A-2-P. This result demonstrates that supplementation with A-2-P can effect increased amidation activity with cultured cells, and that maximal amidation activity is reached at lower concentrations (with a related β-cell line), than the concentrations that increased insulin output (about 400 μM; FIG. 23).

D. Optimal Copper Concentration for PAM Activity

βG 191/26 cell monolayers in T25 flasks were changed to RPMI medium ± copper, or BetaGene Medium ± additional copper (the latter medium contains 5 nM copper). Medium samples were collected after 24 h and the GLP-1 species were separated and quantified by HPLC. The results in FIG. 25 show that supplementing RPMI (which has no copper in its formulation) increases the output of amidated GLP-1. Further supplementation of BetaGene medium with copper to 250 and 500 nM does not increase amidated GLP-1, whereas 1 μM copper tends to decrease amidated GLP-1. These results indicate that 5 nM copper is adequate for PAM activity in cultured neuroendocrine cells. It should be noted that cells in BetaGene Medium have higher output of non-amidated GLP-1, and thus a lower ratio of amidated product than cells with RPMI. Both forms of GLP-1 are active, so this final processing step is less critical for GLP-1 production.

A human cell line, βG H01, was found to naturally express GLP-1. This cell line was used to test the effect of 5 nM copper on amidation. In medium without copper these cells contained 3 ng of GLP-1, with amidated GLP-1 constituting slightly more than half. In the presence of copper the GLP-1 content was increased 4 fold, with amidated GLP-1 constituting more than 80% of the total. This indicates that with conventional culture conditions the same concentration of copper can be used for both rodent and human cells that make an amidated product.

E. Lack of Cytotoxic Effect of A-2-P on Primary Human Islets

Human islets encapsulated in alginate beads were set up in 24 well plates with about 50 islet equivalents/well and cultured in BetaGene Medium with or without added A-2-P and copper. Secretory function and glucose-sensing was determined by incubating the islets with different concentrations of glucose for 90 minutes (from 2.2 to 22 mM). This glucose dose-response test was performed immediately before adding ascorbate to the cultures and at 2 week intervals. In the first 2 weeks 500 µM A-2-P, and 1 µM copper was supplemented. In the second 2 weeks ascorbate was increased to 2 mM, copper was kept at 1 µM. FIG. 26 shows that A-2-P did not impair function as indicated by sensing of glucose, (EC50 for control and A-2-P islets was the same). Additionally, the maintenance of maximal insulin secretion indicates that there is minimal toxicity of A-2-P for these culture times.

The above findings demonstrate the stability of A-2-P in media, the effectiveness of A-2-P in supporting amidation-activity in cell culture, the beneficial effect on secretory function, and the concomitant lack of cytotoxicity with long-term cultures of neuroendocrine cell lines and primary human islets.

EXAMPLE 12

Altering Responsiveness of Human Neuroendocrine Cell Lines to Modulators of Secretion

βG 498/20 cells secrete insulin from a regulated secretory pathway, as evidenced by an approximate 12-fold increase in basal insulin secretion versus that stimulated by PMA, carbachol, or a stimulatory cocktail (Swiss). Also, there is a lack of responsiveness to glucose and glucose plus IBMX. A preferred embodiment for the cell-based delivery of insulin includes the capacity to modulate release of the peptide in response to post-prandial (such as glucose) and/or hypoglycemic signals. The pancreatic β-cell senses a variety of extracellular molecules through metabolism, receptors, and ion channels. Each of these sensing mechanisms impacts intracellular calcium levels, with increases in this ion stimulating the release of insulin.

Two lines a experimental evidence implicate Ca^{2+} in regulated insulin secretion from βG 498/20 cells: firstly, PMA and carbachol each exert effects on secretion via the stimulation of protein kinase C, where "C" is an abbreviation for "calcium"; and secondly, verapamil partially inhibits stimulated secretion from βG 498/20 cells. Verapamil antagonizes the uptake of extracellular Ca^{2+}. Efforts are underway to exploit the role of Ca^{2+}-regulated secretion in βG 498/20 cells, and to engineer these cell lines to respond to a variety of secretory modulators that are known to be involved in the physiological regulation of insulin secretion from the pancreatic beta cell. Table 12 below lists potential candidates for engineering and the molecules to which they respond.

TABLE 12

Candidate Transgenes for Altering Secretory Responses of Neuroendocrine Cell Lines.

Candidate Transgenic Protein	Responsive To:
glucokinase	glucose
GLUT-2 transporter	glucose
SUR	ATP, diazoxide
Kir	ATP
late rectifying K channels	membrane polarity, potassium
calcium channels	membrane polarity, calcium
GLP-1 receptor	GLP-1
muscarinic receptor	acetyl choline
pancreatic polypeptide receptor	pancreatic polypeptide
somatostatin receptor	somatostatin
alpha 2 adrenergic receptor	epinephrine
leptin receptor	leptin

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS:

1. A method for preparing an immortal human neuroendocrine cell that secretes at least a first polypeptide, comprising inducing a non-immortal human neuroendocrine cell to proliferate by initial infection with a recombinant adenovirus, and providing to the proliferating non-immortal human neuroendocrine cell an effective amount of at least a first immortalizing genetic construct by infection with at least a first recombinant retrovirus that comprises said at least a first recombinant immortalizing genetic construct, said construct comprising at least a first immortalizing expression unit under the transcriptional control of a neuroendocrine cell specific promoter, the construct expressing at least a first immortalizing product in said cell.

2. The method of claim 1, wherein said non-immortal human neuroendocrine cell is a fetal cell, a primary cell obtained from human tissue or a cell obtained from a human neuroendocrine tumor.

3. The method of claim 1, wherein said human neuroendocrine cell is a cell type listed in Table 1.

4. The method of claim 3, wherein said human neuroendocrine cell is a pancreatic β-cell or a pituitary cell.

5. The method of claim 1, wherein the secretion of at least a first polypeptide from said human neuroendocrine cell is regulated by at least a first modulator.

6. The method of claim 1, wherein said human neuroendocrine cell secretes at least a first endogenous secretory polypeptide.

7. The method of claim 1, wherein said human neuroendocrine cell is provided with at least a first exogenous gene that encodes at least a first exogenous secretory polypeptide and wherein said cell secretes said at least a first exogenous secretory polypeptide.

8. The method of claim 1, wherein said human neuroendocrine cell secretes at least a first secretory polypeptide listed in Table 1, Table 2, Table 3, Table 4 or Table 5.

9. The method of claim 1, wherein said human neuroendocrine cell secretes at least a first glycosylated polypeptide, at least a first amidated polypeptide, at least a first hormone, at least a first growth factor or at least a first enzyme.

10. The method of claim 1, wherein said human neuroendocrine cell is grown in defined media.

11. The method of claim 10, wherein said defined media is BetaGene media as set forth in Table 9.

12. The method of claim 1, wherein said human neuroendocrine cell is induced to proliferate by initial infection with at least a first recombinant adenovirus that comprises at least a first gene that induces cellular proliferation, the gene under the control of a promoter specific for said neuroendocrine cell.

13. The method of claim 12, wherein said human neuroendocrine cell is induced to proliferate by initial infection with at least a first adenovirus comprising at least a first gene encoding a temperature sensitive large T antigen, IGF-1, IGF-1 receptor, growth hormone, growth hormone receptor, IRS-2 or insulin receptor.

14. The method of claim 1, wherein said at least a first immortalizing genetic construct expresses at least a first protein that perturbs signal transduction, at least a first proto-oncogene, oncogene or mutant tumor suppressor product listed in Table 8.

15. The method of claim 14, wherein said at least a first immortalizing genetic construct expresses at least a first component of telomerase.

16. The method of claim 15, wherein said at least a first immortalizing genetic construct expresses at least a first telomerase catalytic component (TERT).

17. The method of claim 14, wherein said at least a first immortalizing genetic construct expresses at least a first temperature sensitive large T antigen, a papillomavirus E6 or a papillomavirus E7.

18. The method of claim 1, wherein said promoter is a promoter listed in Table 1.

19. The method of claim 1, wherein said promoter is a modified promoter having increased transcriptional activity.

20. The method of claim 19, wherein said promoter comprises multimerized promoter elements.

21. The method of claim 20, wherein said promoter comprises multimerized insulin gene promoter elements.

22. The method of claim 21, wherein said promoter comprises multimerized RIP elements.

23. The method of claim 22, wherein said promoter comprises the DNA sequence of SEQ ID NO:11.

24. The method of claim 1, wherein the expression of said at least a first immortalizing product in said cell is conditional on temperature or on at least a first exogenously added component.

25. The method of claim 1, wherein said at least a first immortalizing genetic construct is separated from said immortal human neuroendocrine cell after immortalization of said cell.

26. The method of claim 1, wherein the starting, non-immortal human neuroendocrine cell is passaged *in vivo*.

27. The method of claim 1, wherein the resultant, immortalized human neuroendocrine cell is passaged *in vivo*.

28. The method of claim 1, wherein said immortal human neuroendocrine cell is grown in contact with a solid support.

29. The method of claim 1, wherein said immortal human neuroendocrine cell is comprised within a bioreactor.

30. The method of claim 1, wherein said immortal human neuroendocrine cell is formulated in a pharmaceutically acceptable medium.

31. The method of claim 1, wherein said immortal human neuroendocrine cell is encapsulated in a biocompatible coating or implantable device.

32. An immortal human neuroendocrine cell prepared by the method of any one of claims 1 to 31.

33. Use of the immortal human neuroendocrine cell as set forth in claim 32 in the preparation of an implantable device.

34. Use of the immortal human neuroendocrine cell as set forth in claim 32 in the preparation of a medicament to provide glucose-responsive insulin secreting capability to an animal.

35. A use according to claim 34, wherein said population of cells are encapsulated in a biocompatible coating.

36. A use according to claim 34, wherein said cells secrete human insulin and wherein said animal is a human subject.

37. A use according to claim 34, wherein said animal has or is suspected of having pre-clinical diabetes.

38. A use according to claim 34, wherein said animal has or is at risk for developing overt diabetes.

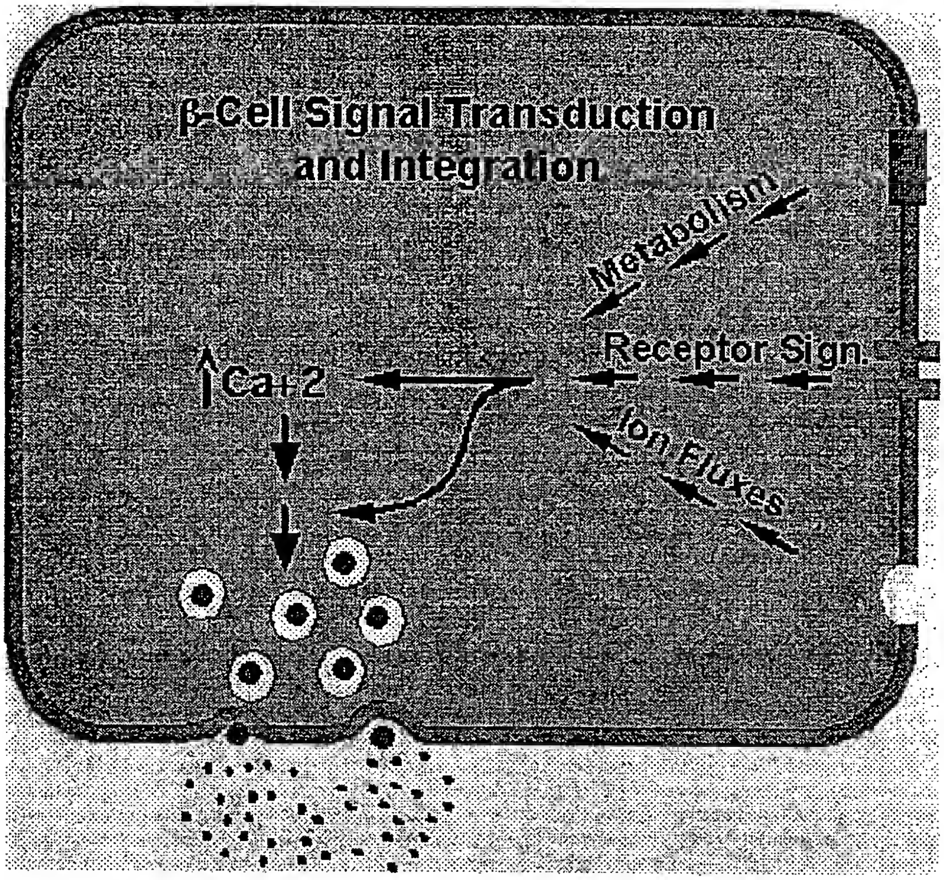

β-Cell Signal Transduction and Integration
Metabolism
Receptor Sign.
Ion Fluxes
↑Ca+2

FIG. 1

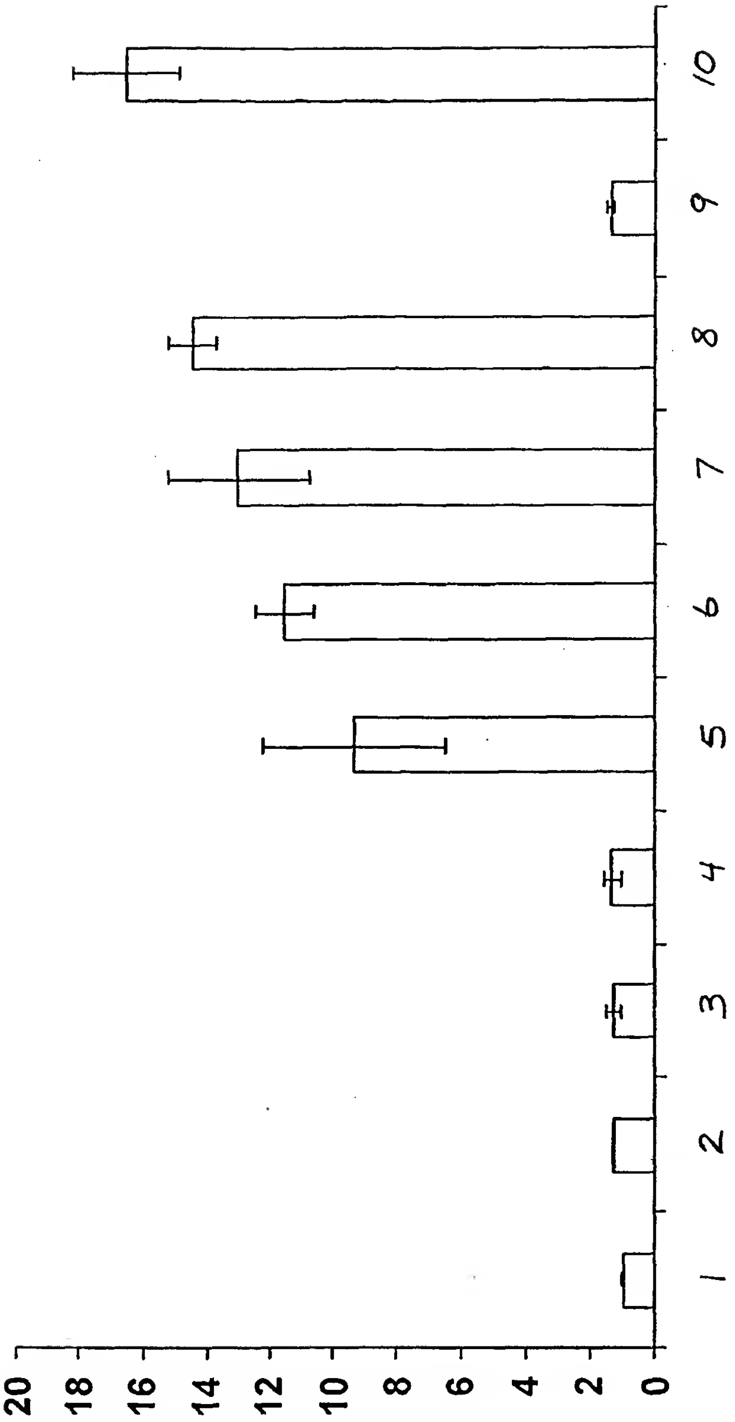


FIG. 2A

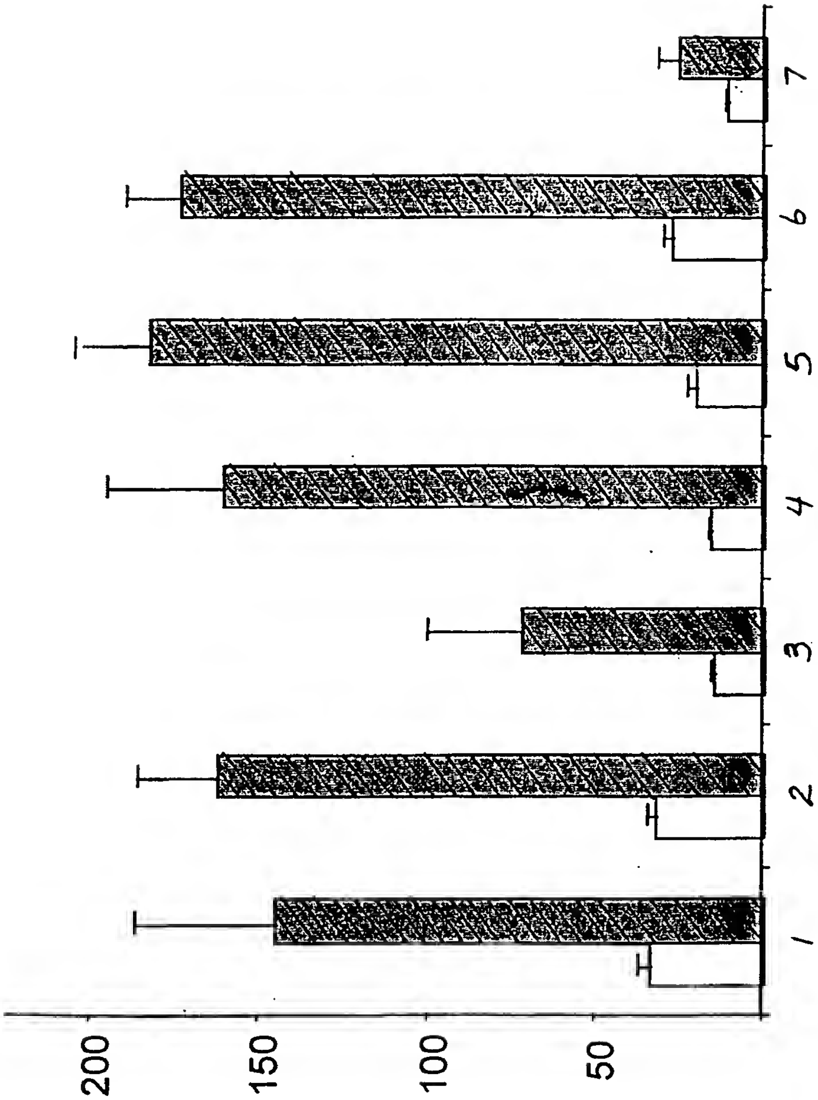


FIG. 2B

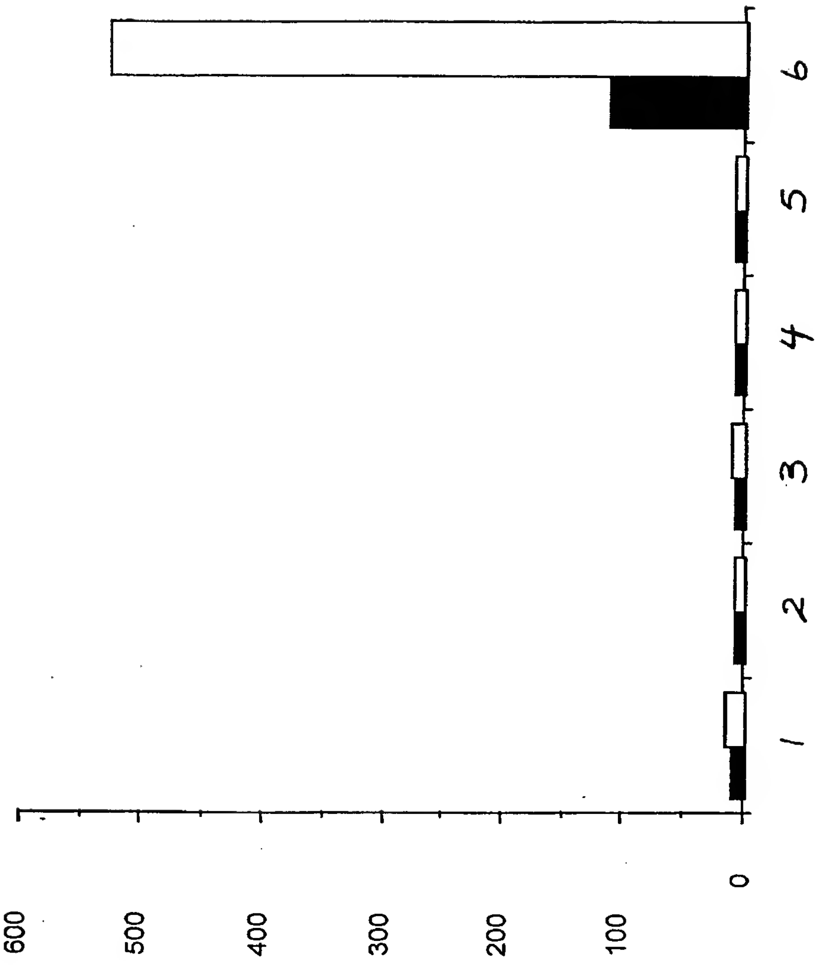


FIG. 3

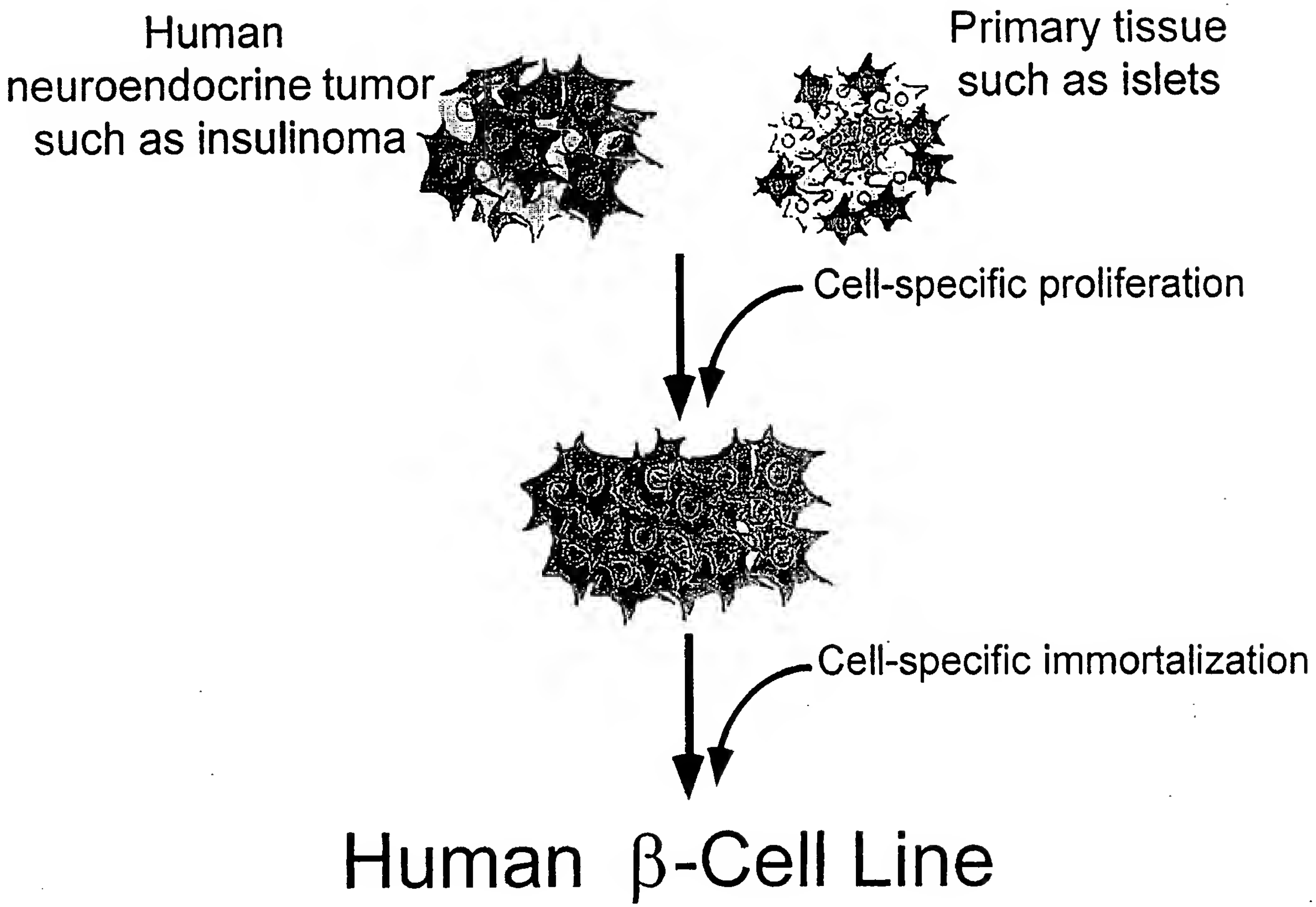

Human
neuroendocrine tumor
such as insulinoma
Primary tissue
such as islets
Cell-specific proliferation
Cell-specific immortalization
Human β-Cell Line

FIG. 4

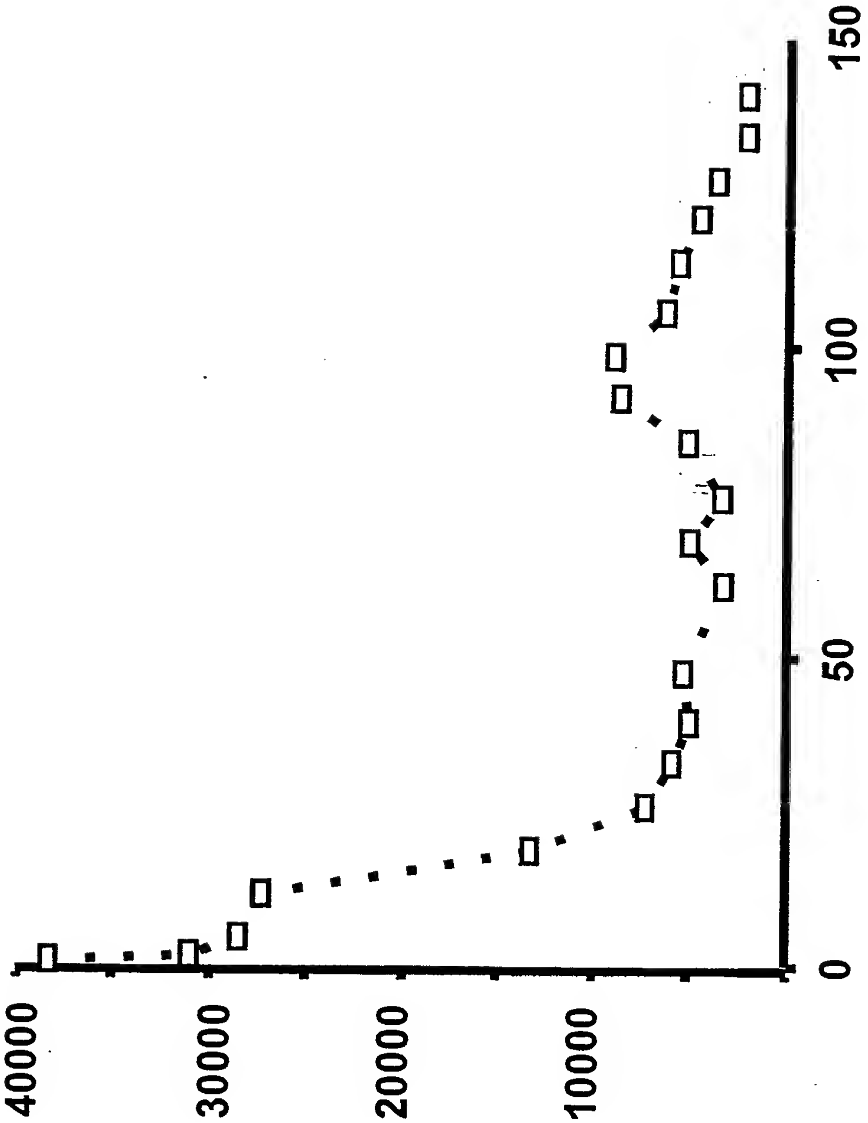


FIG. 5

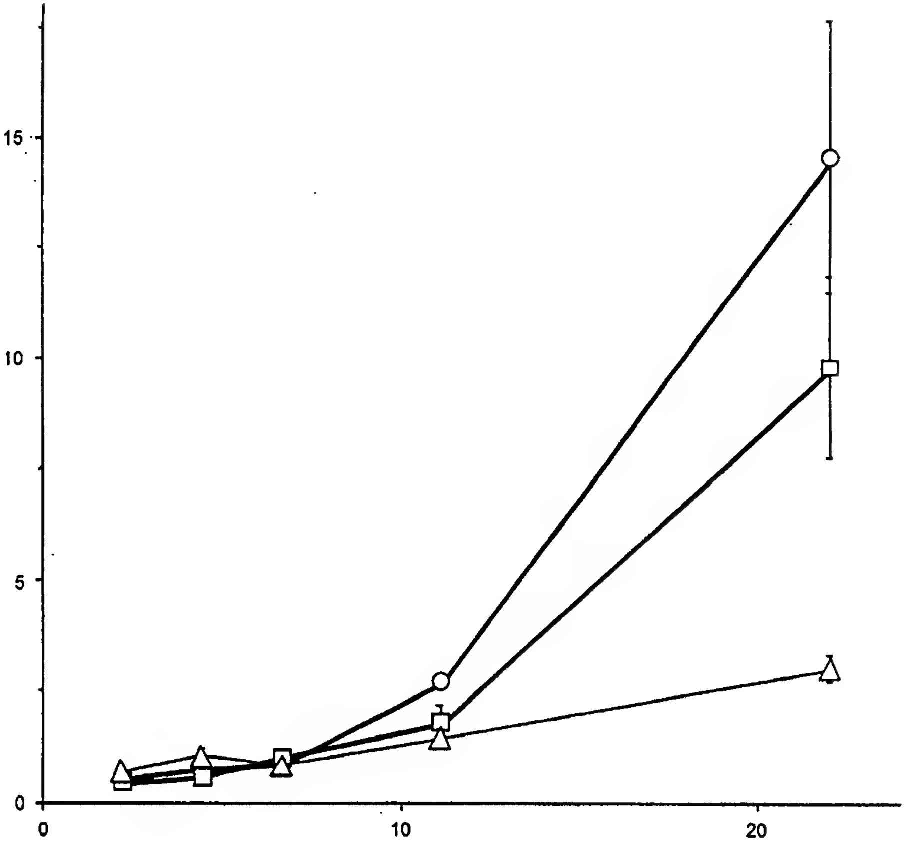


FIG. 6

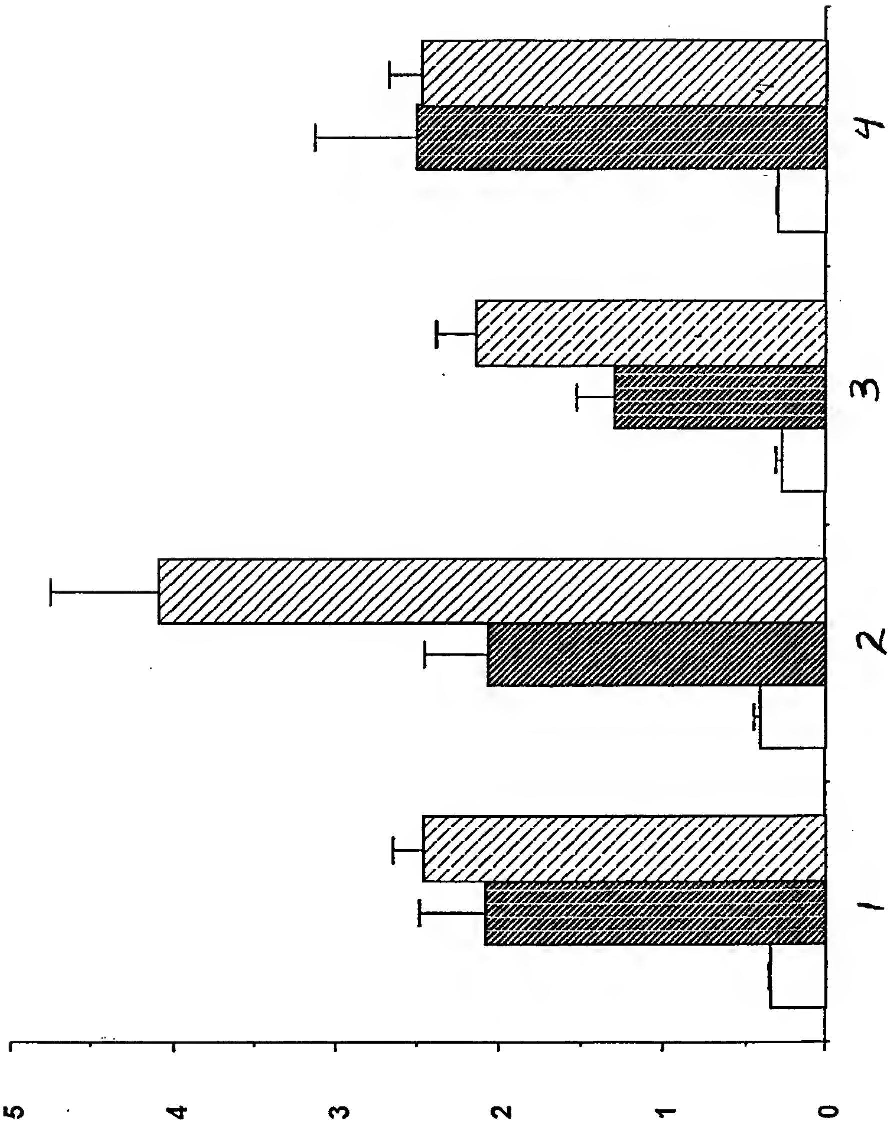


FIG. 7

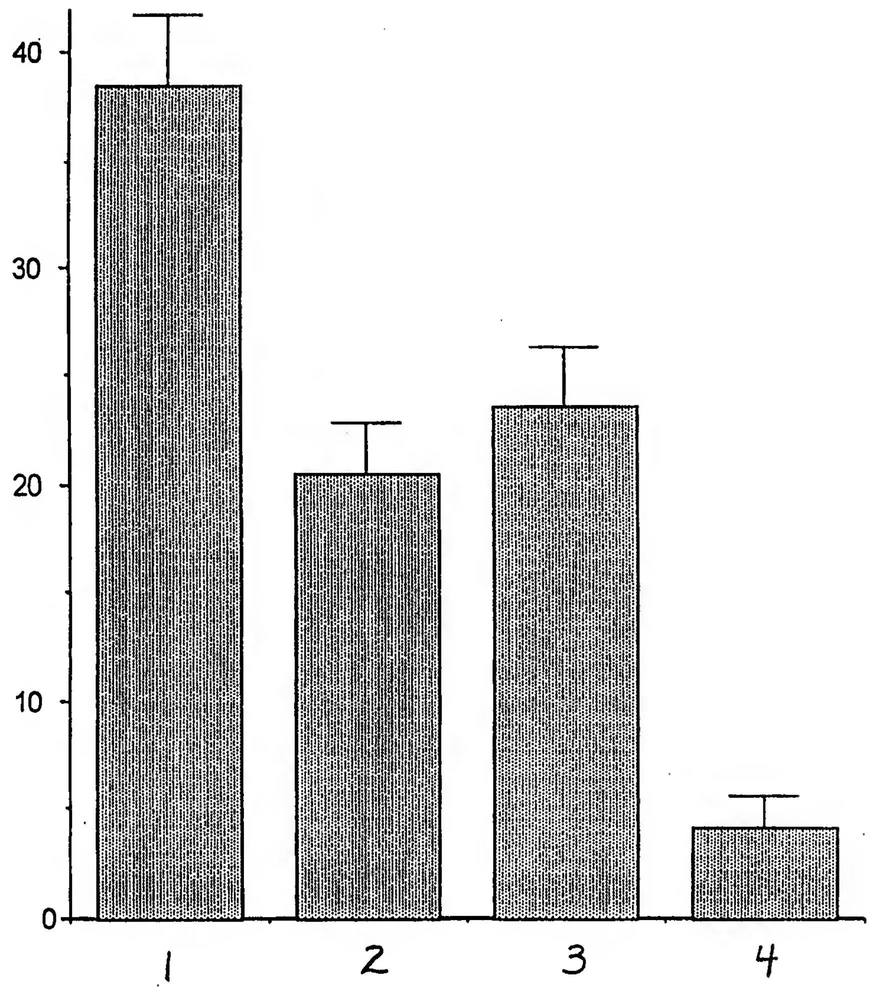


FIG. 8

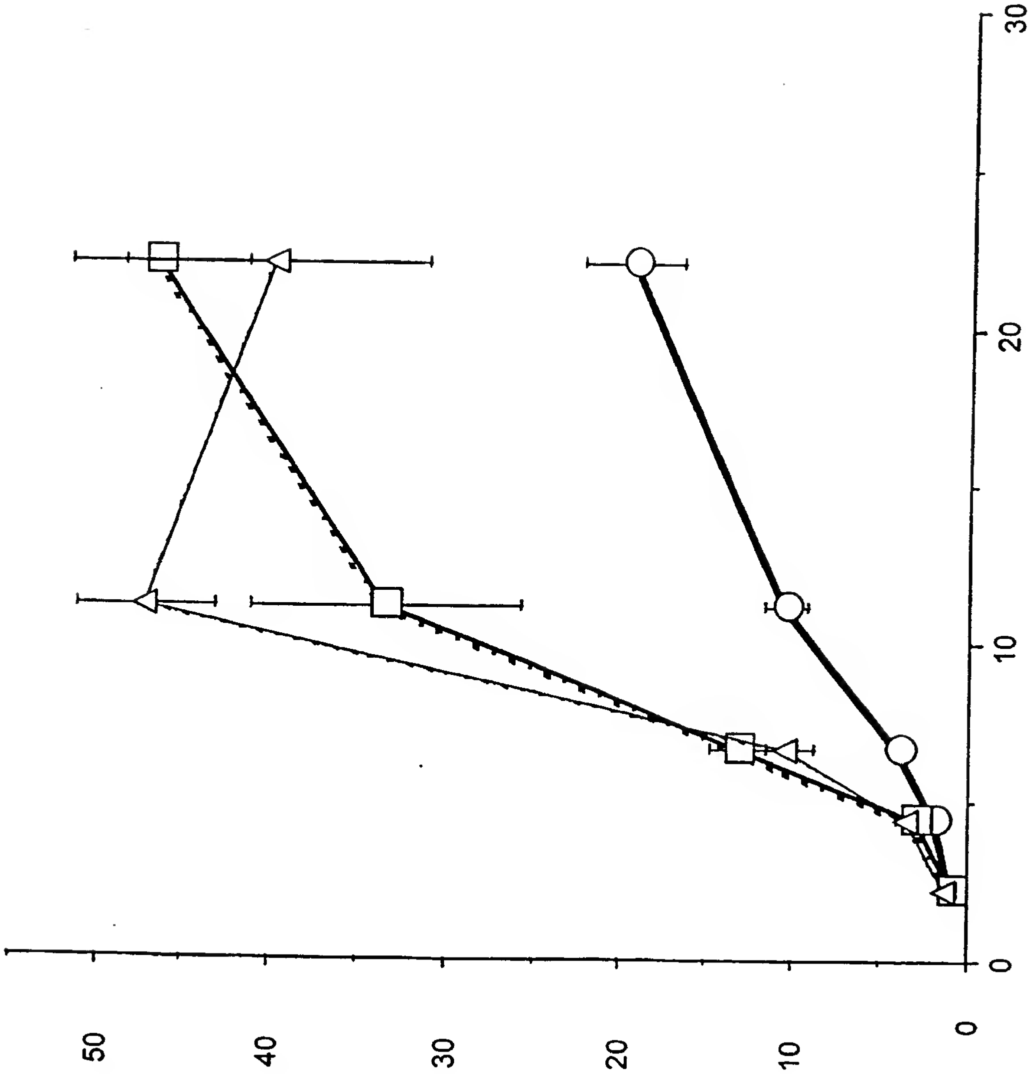


FIG. 9

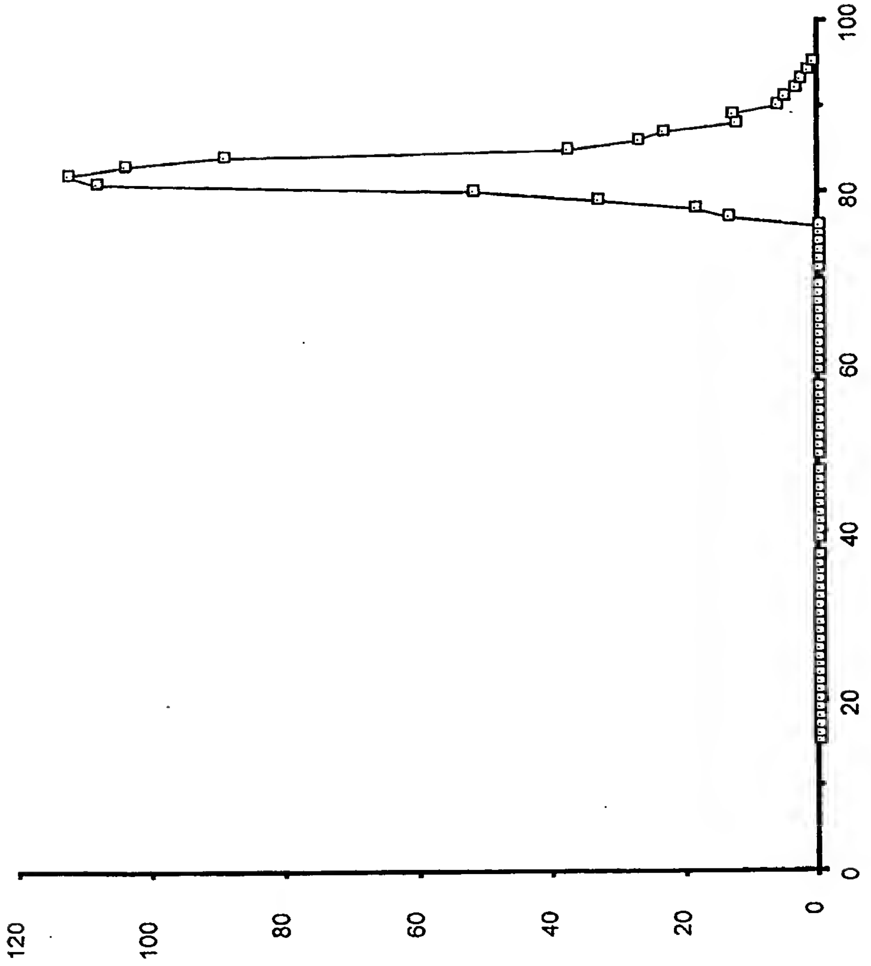


FIG. 10A

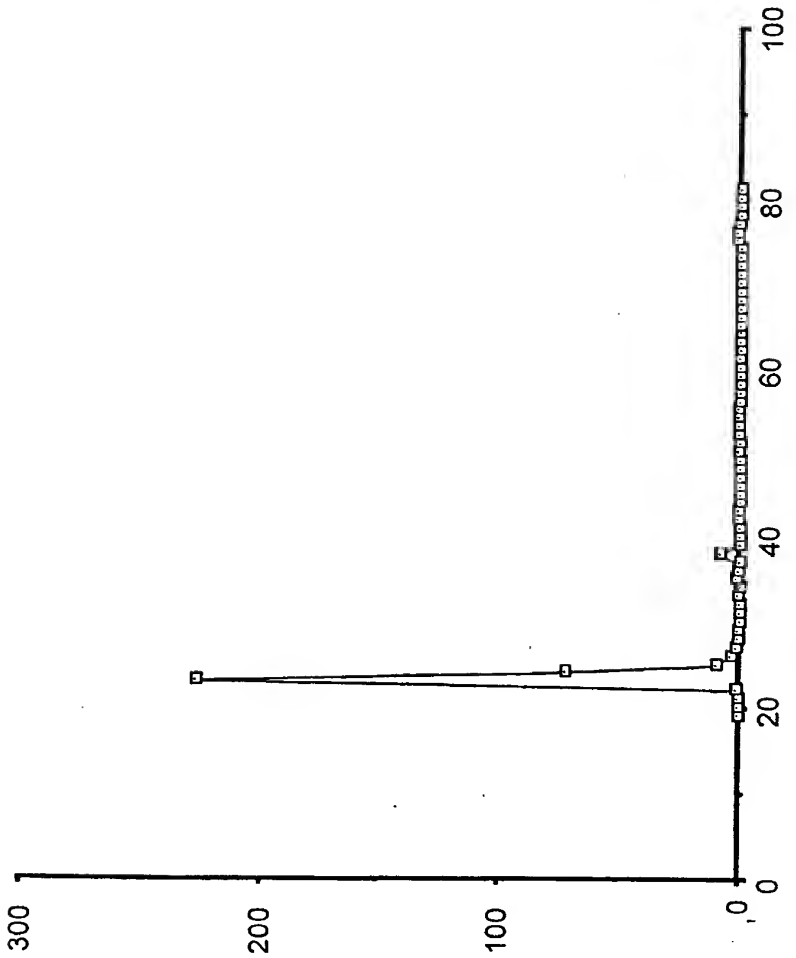
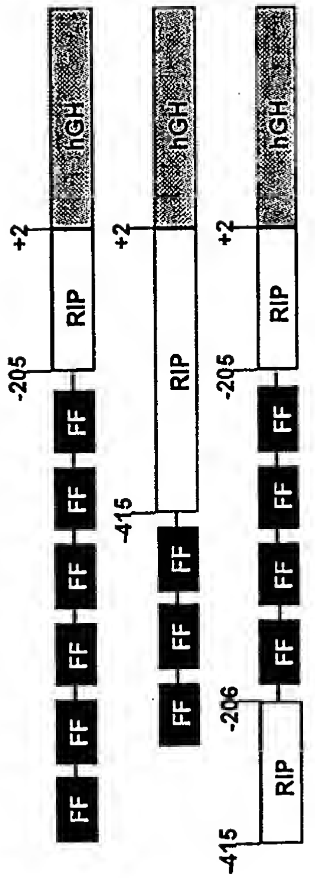


FIG. 10B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FF
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RIP
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FIG. 11A

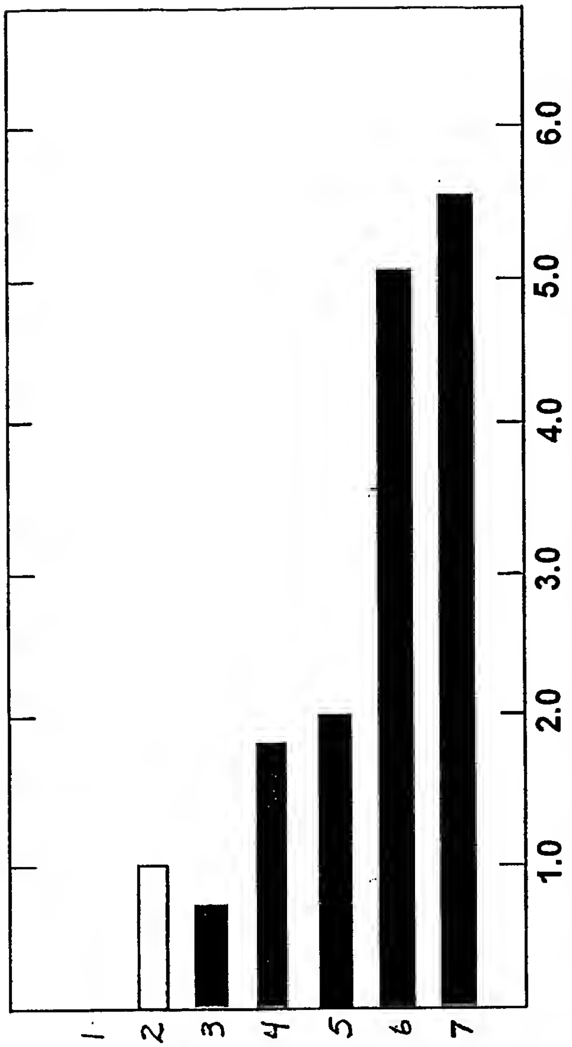


FIG. 11B

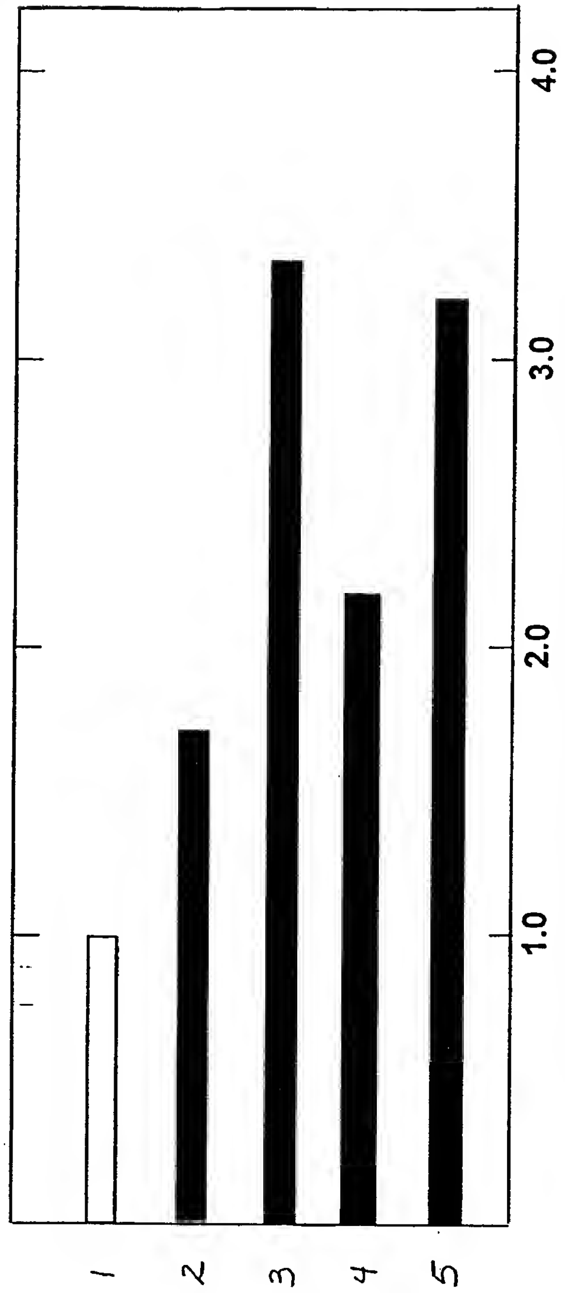


FIG. 12A

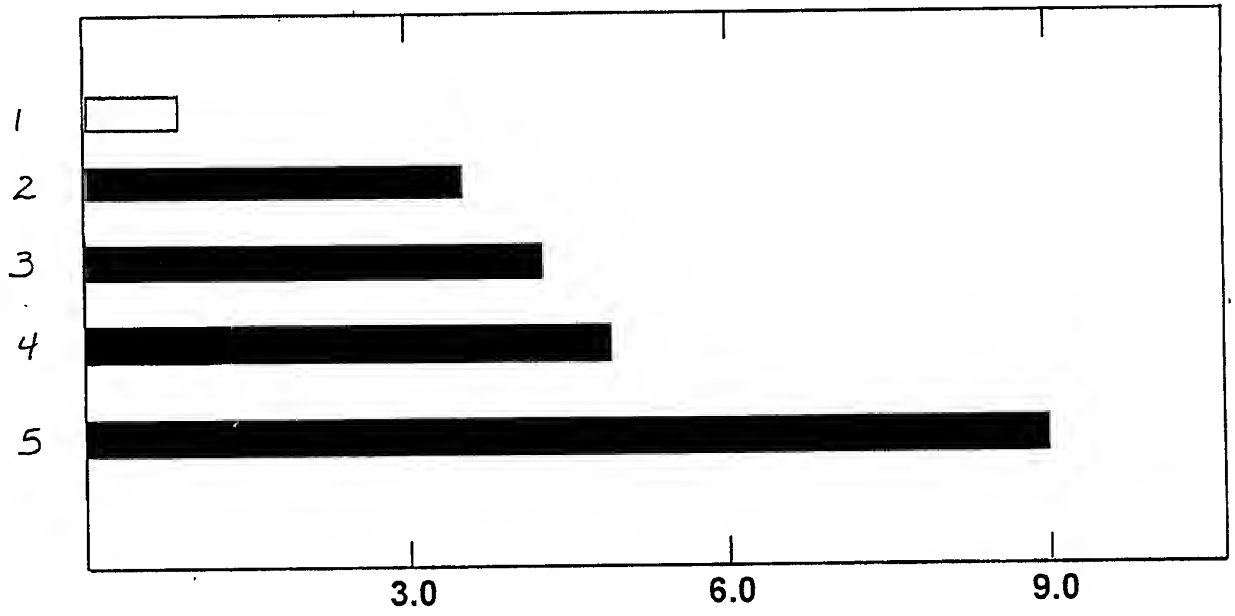


FIG. 12B

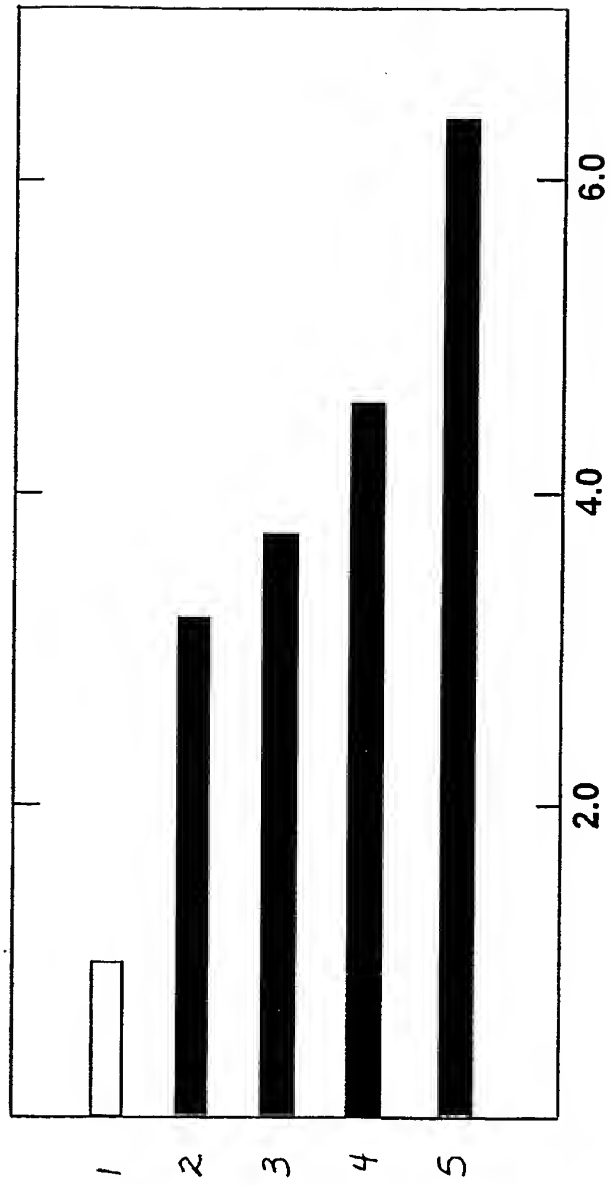


FIG. 12C

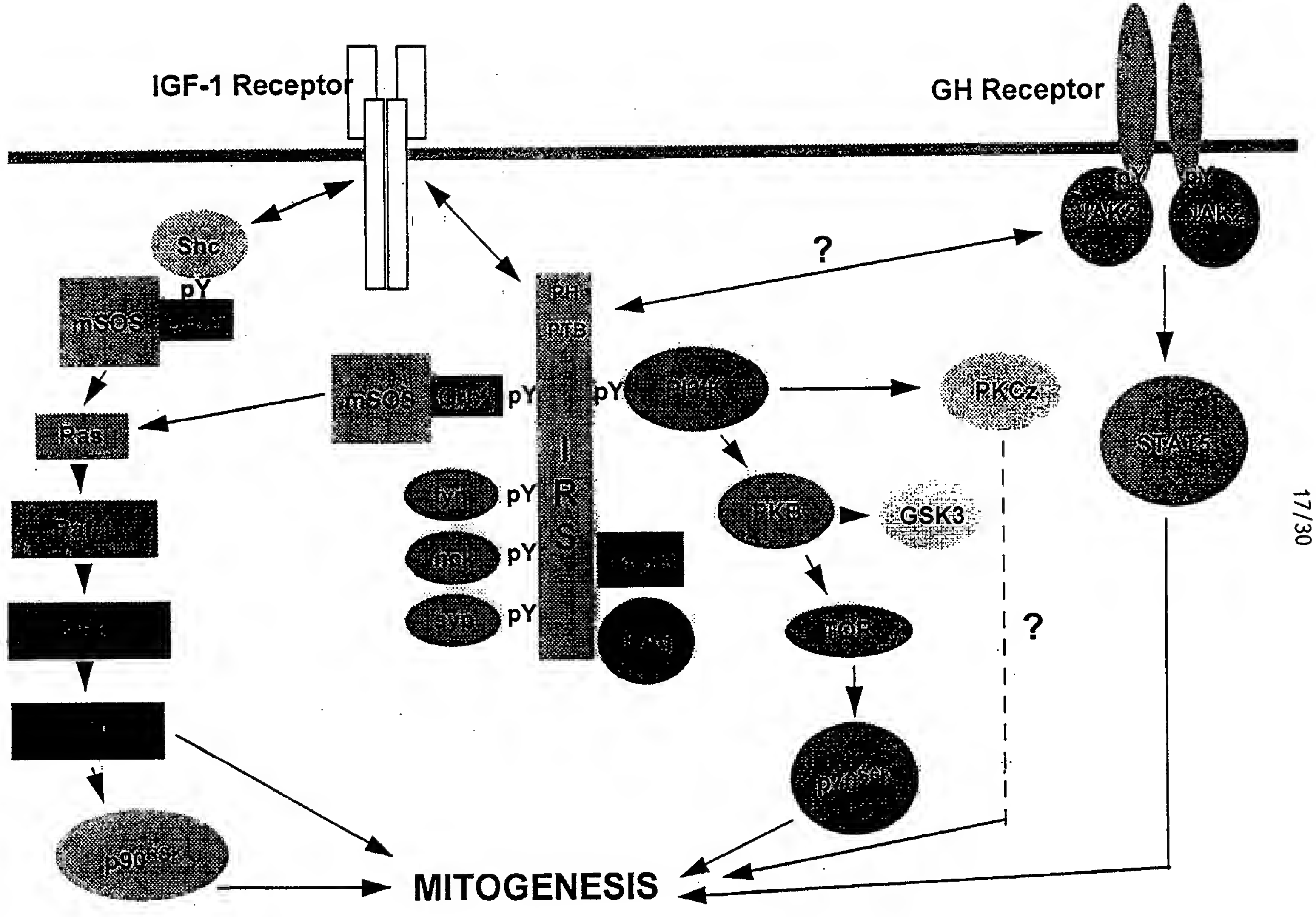

IGF-1 Receptor
GH Receptor
Shc
pY
mSOS
mSOS
Ras
PH
PTB
pY
pY
pY
pY
pY
I
R
S
PI3K
PKCz
PKB
GSK3
JAK2
JAK2
STAT5
?
?
MITOGENESIS

FIG. 13

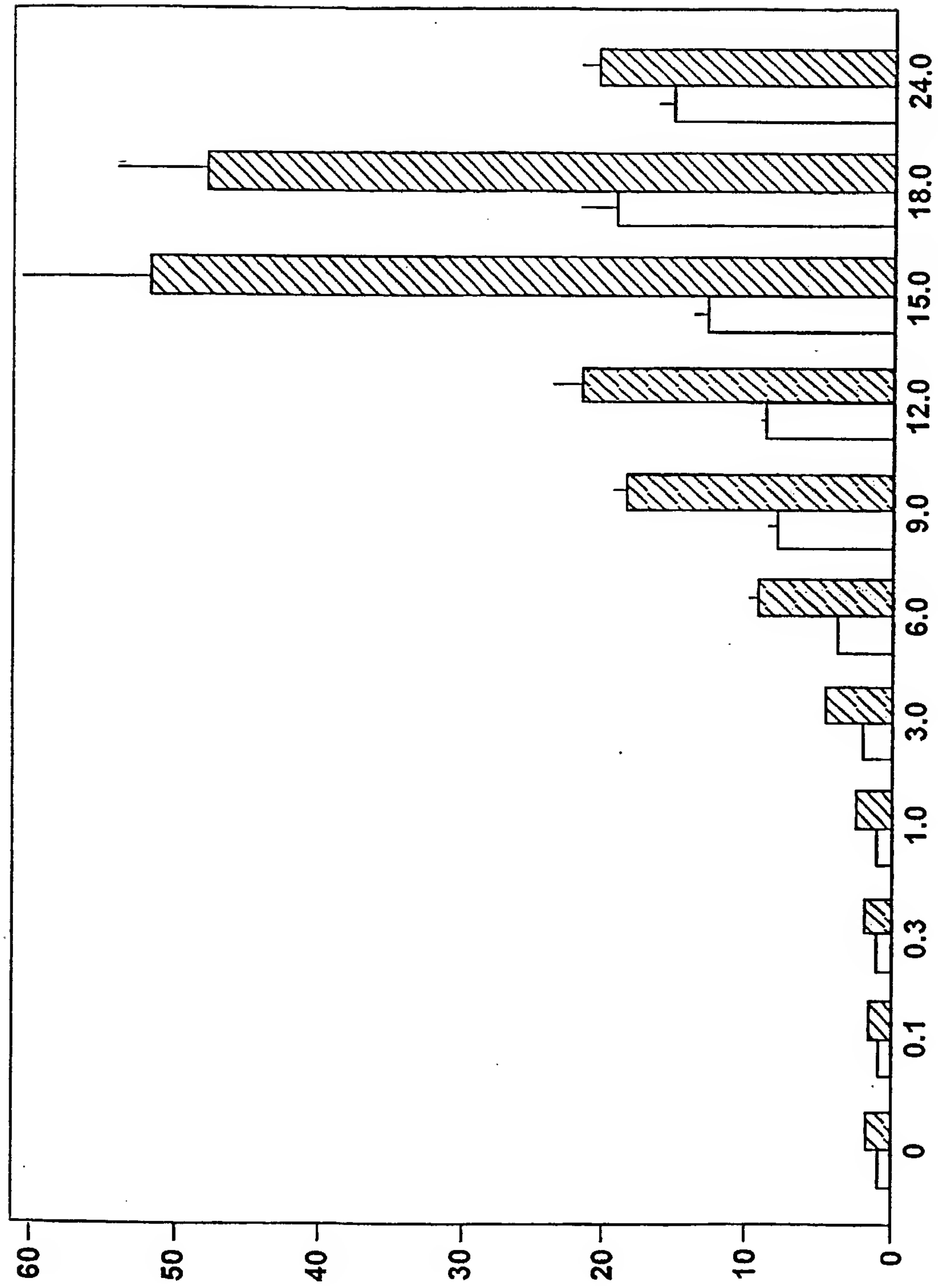


FIG. 14

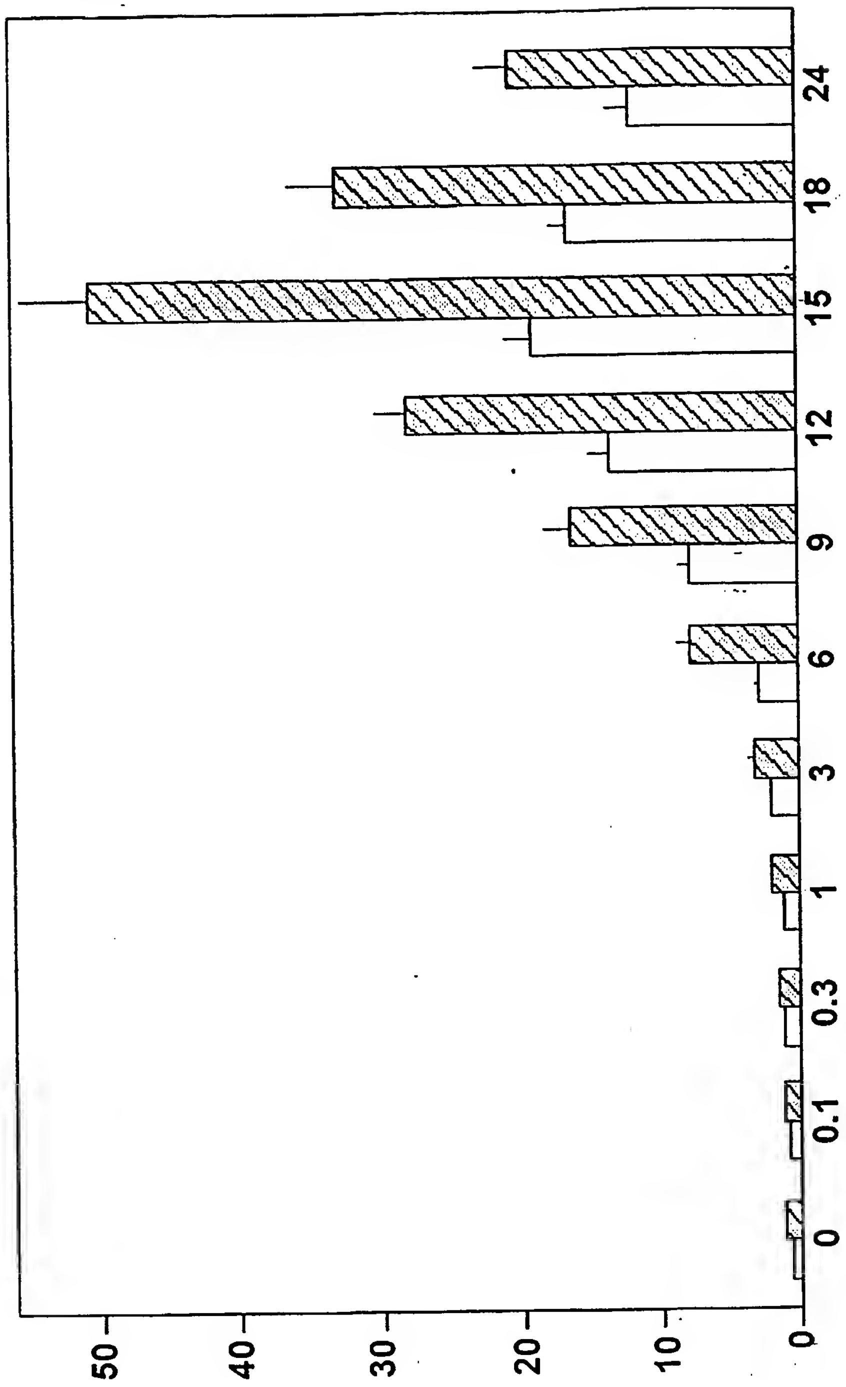


FIG. 15

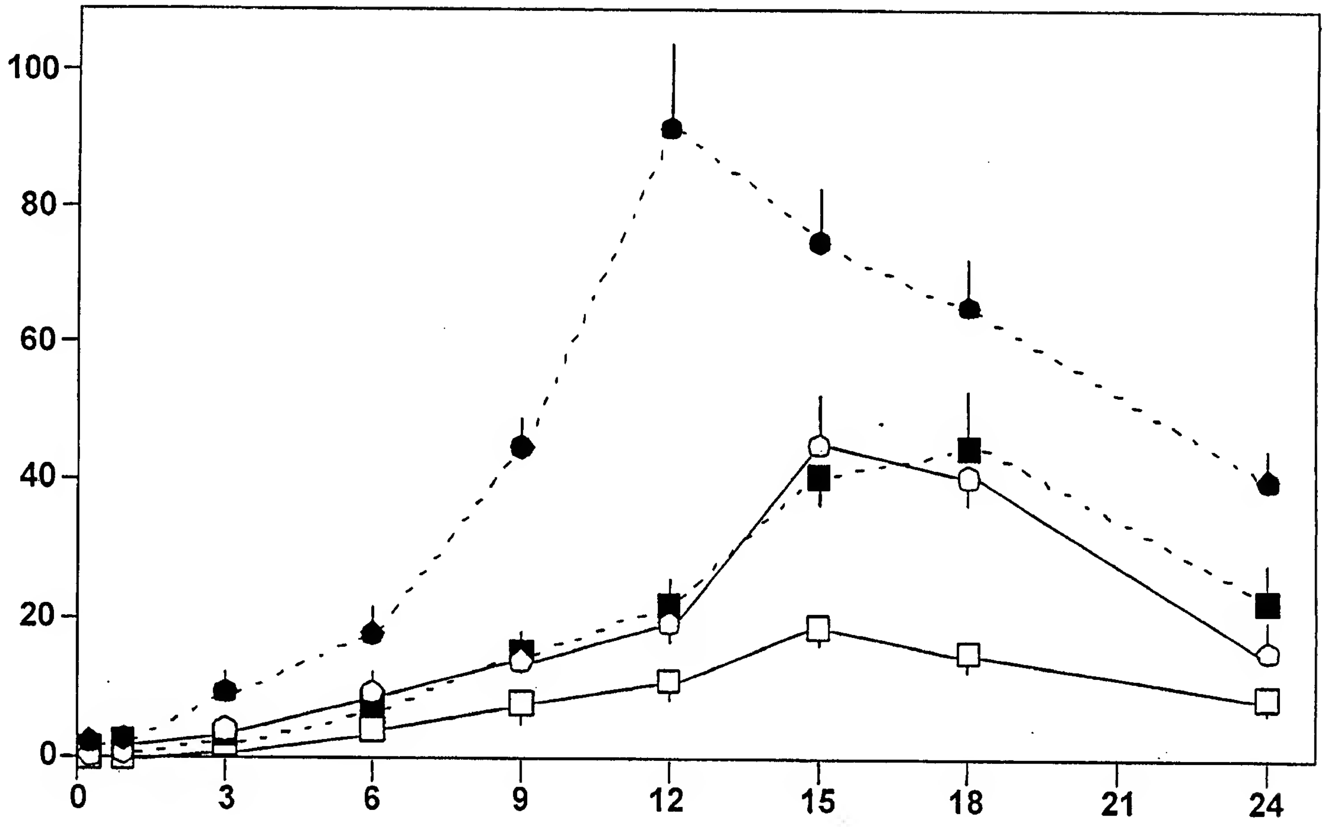


FIG. 16

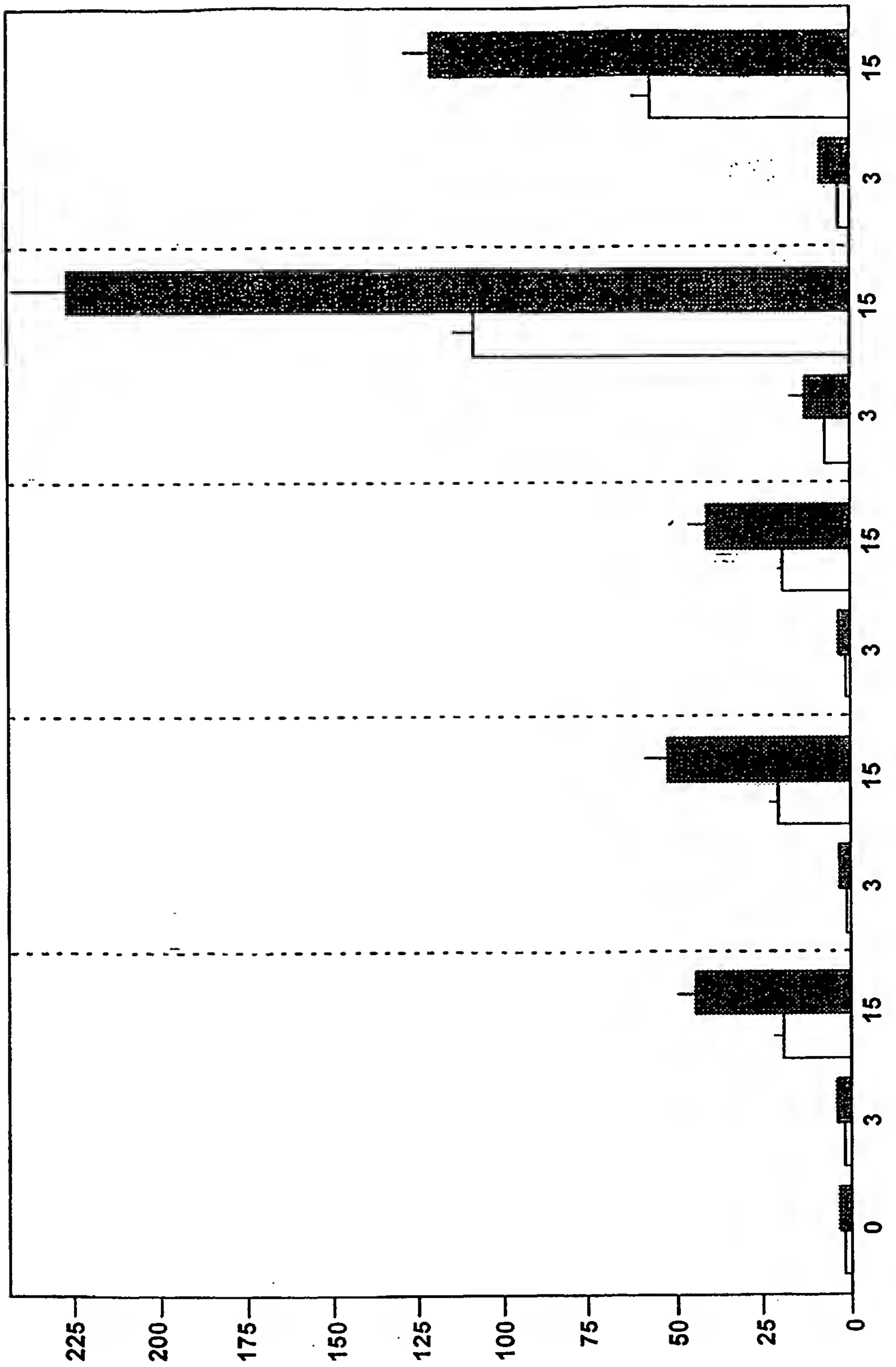


FIG. 17A

FIG. 17B

FIG. 17C

FIG. 17D

FIG. 17E

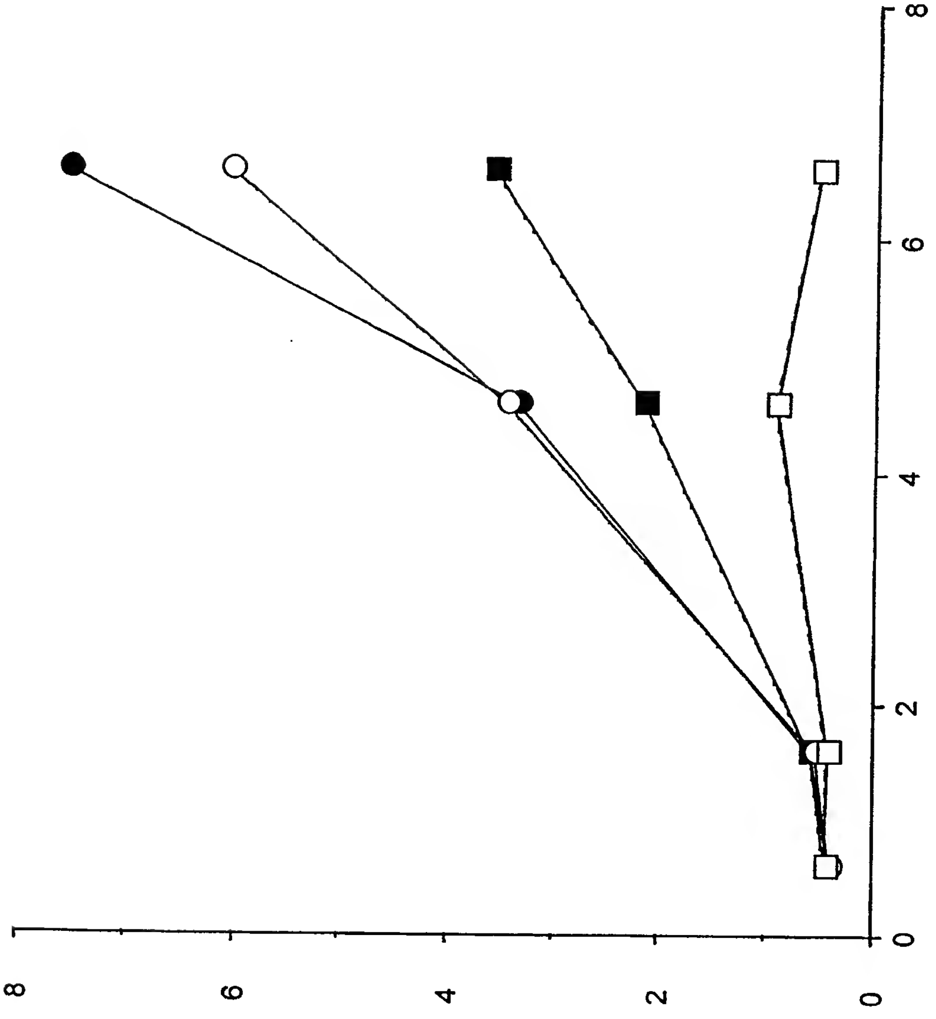
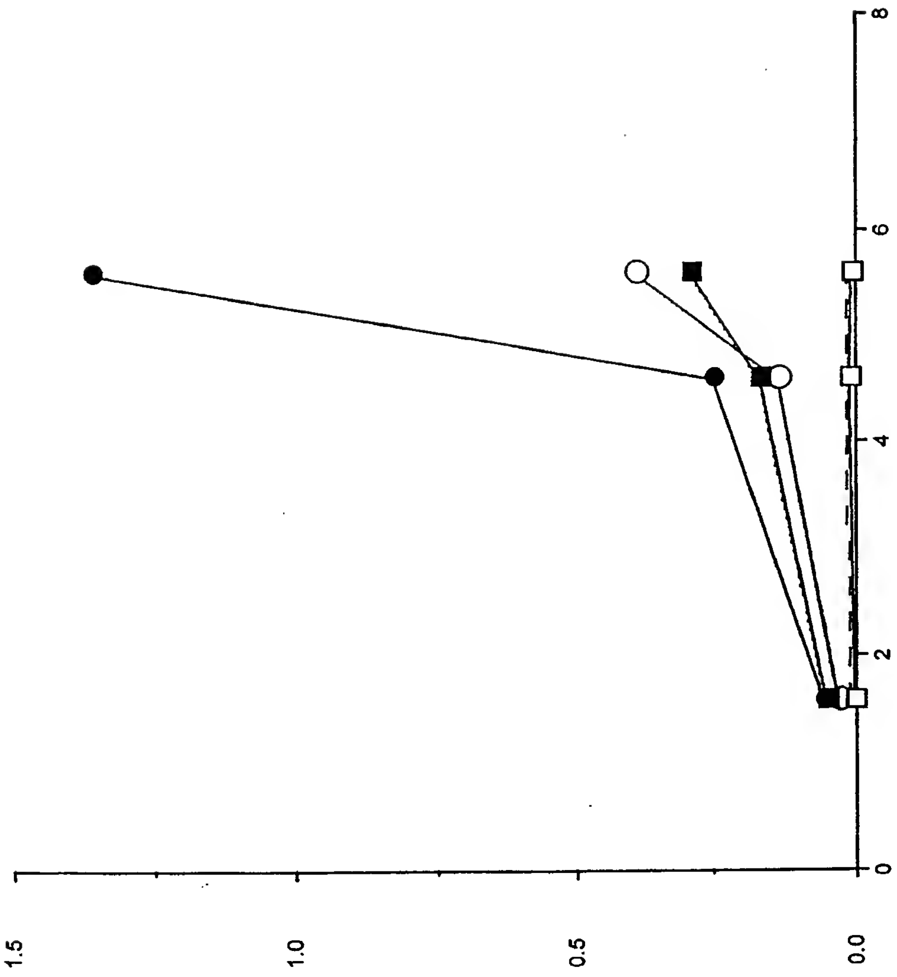


FIG. 18



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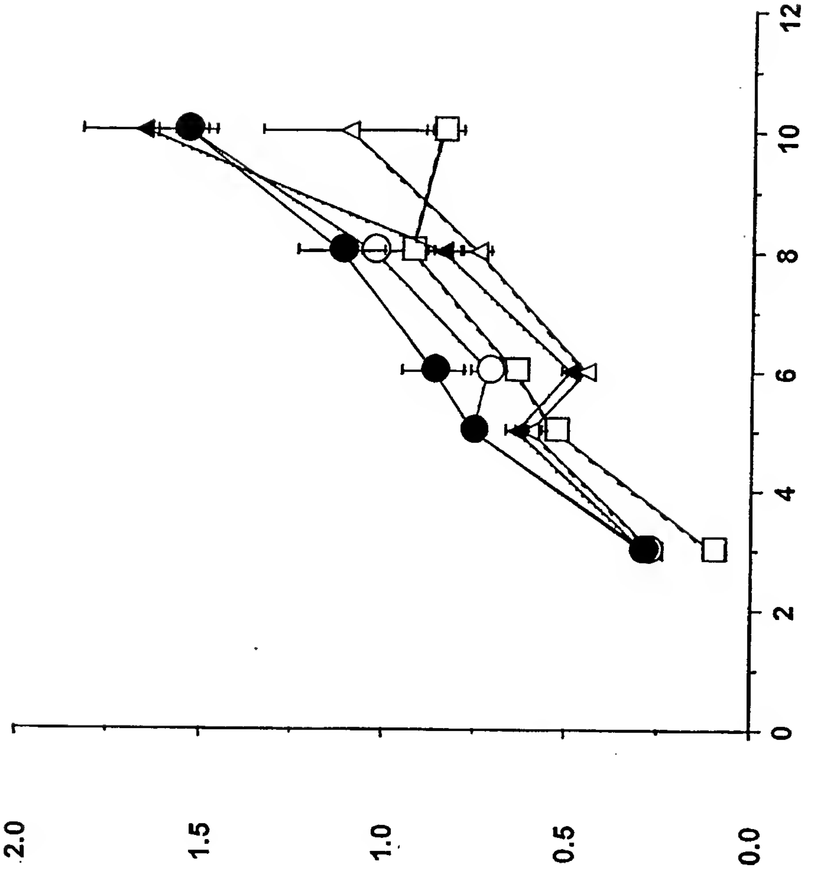
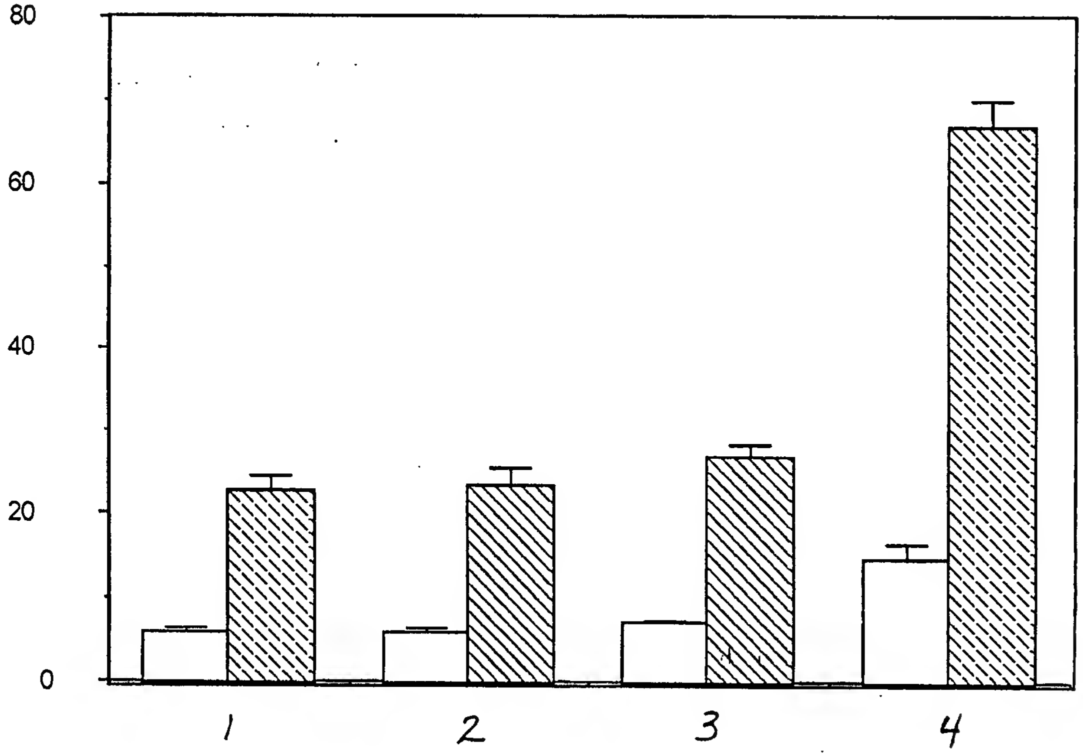


FIG. 20



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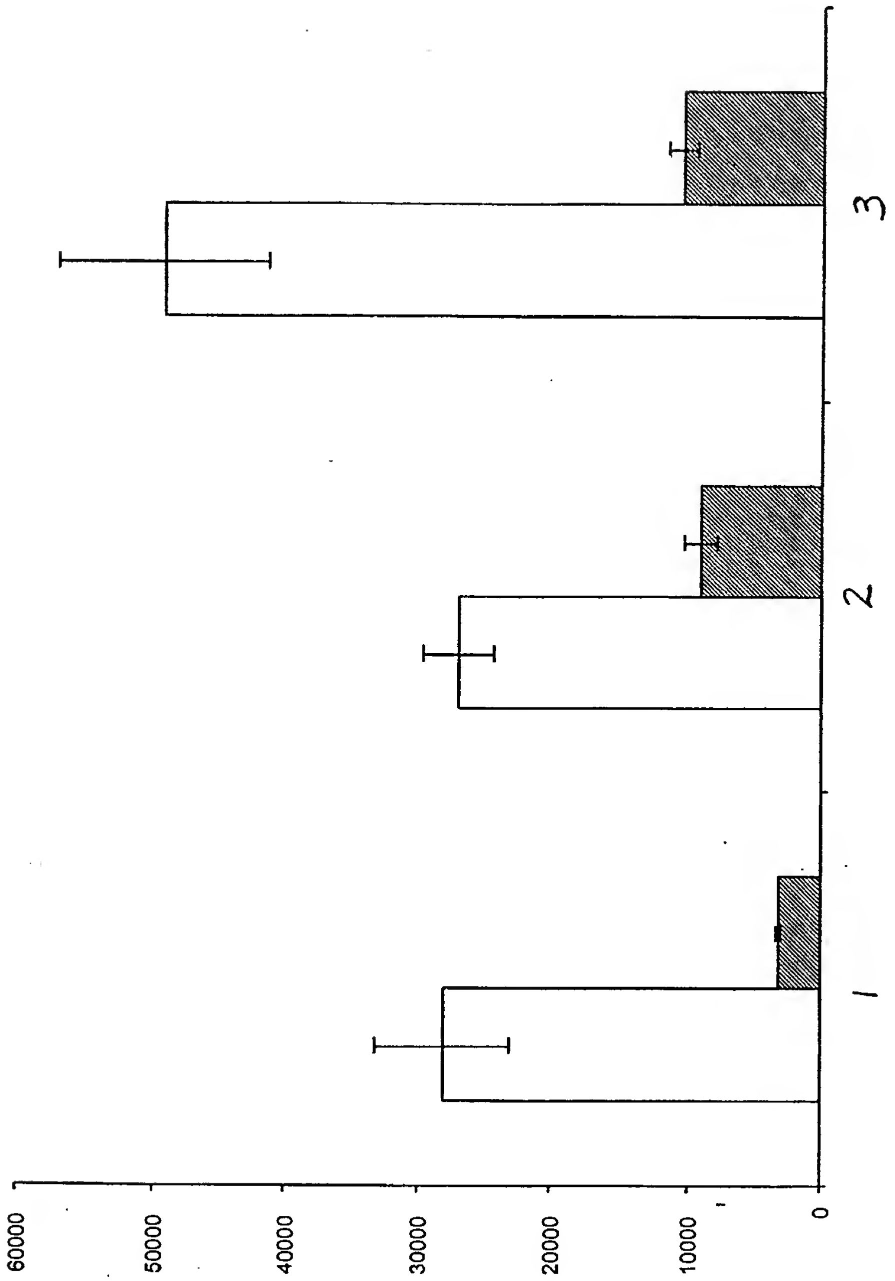
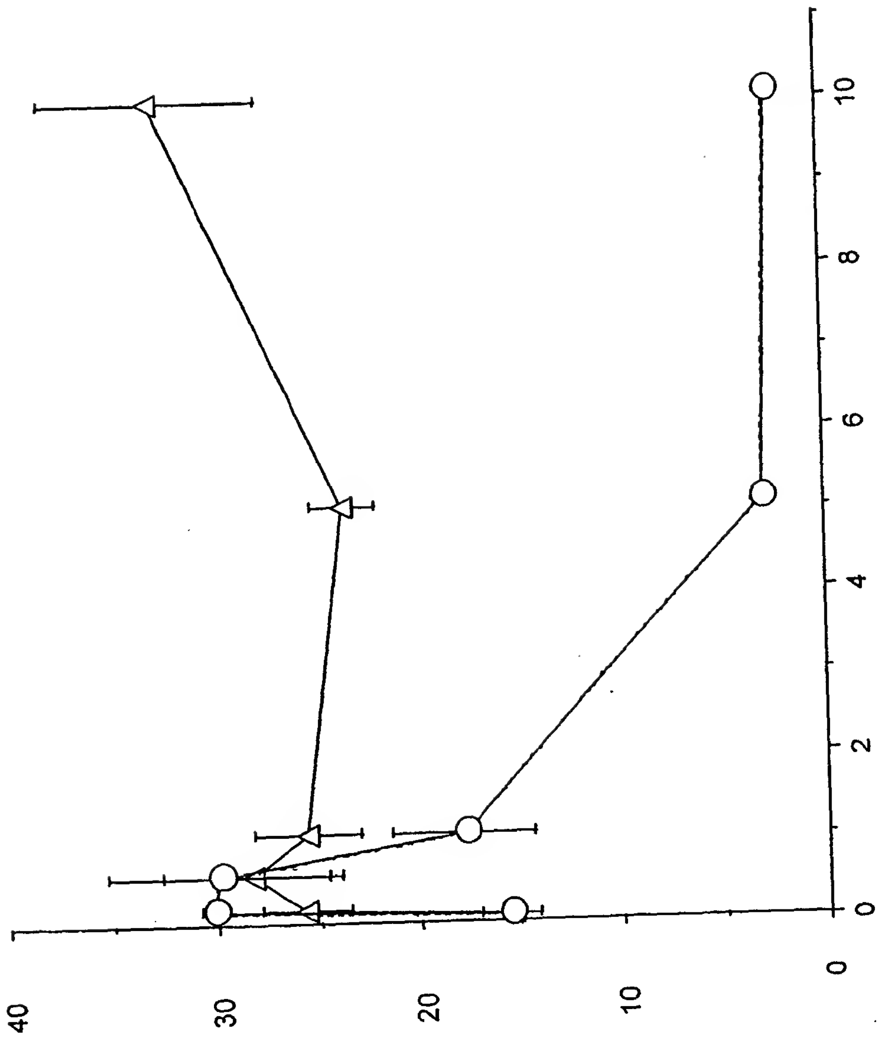


FIG. 22



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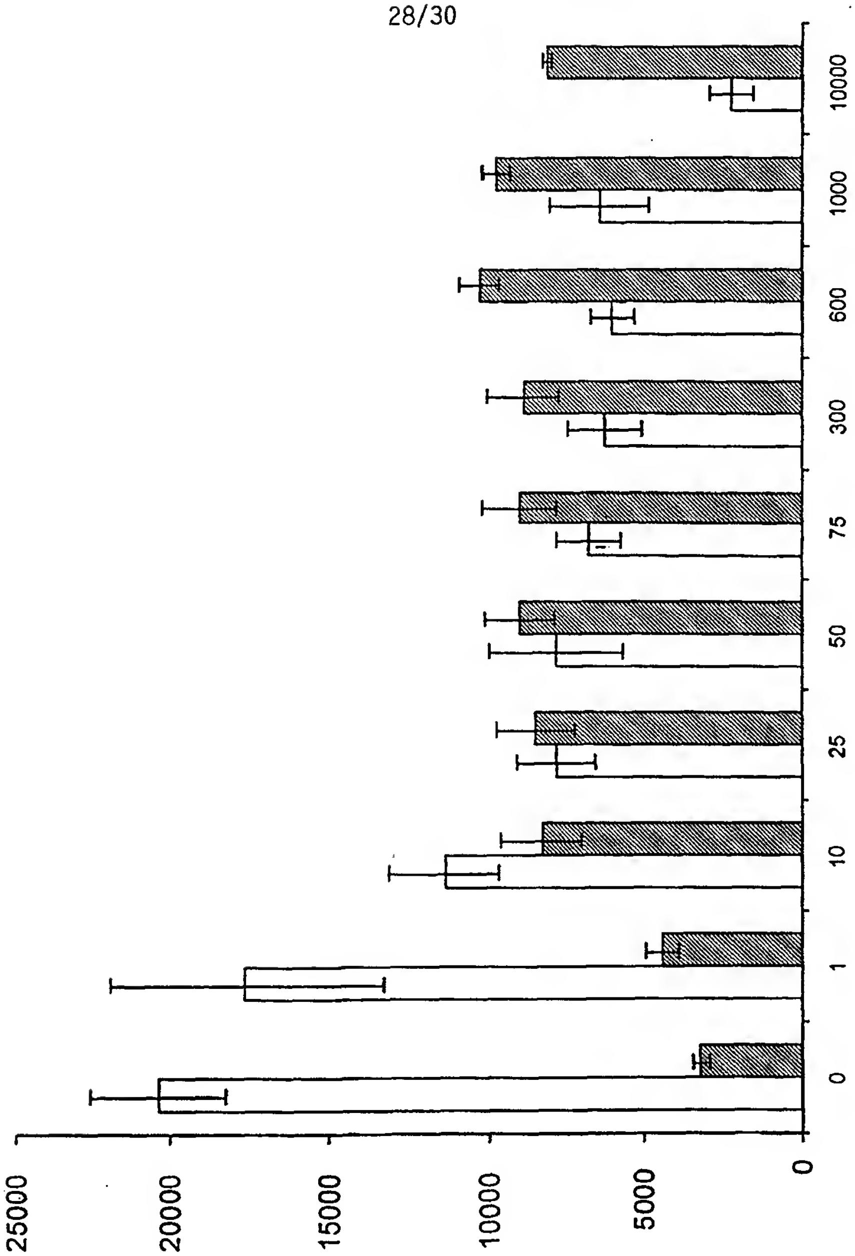
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FIG. 24

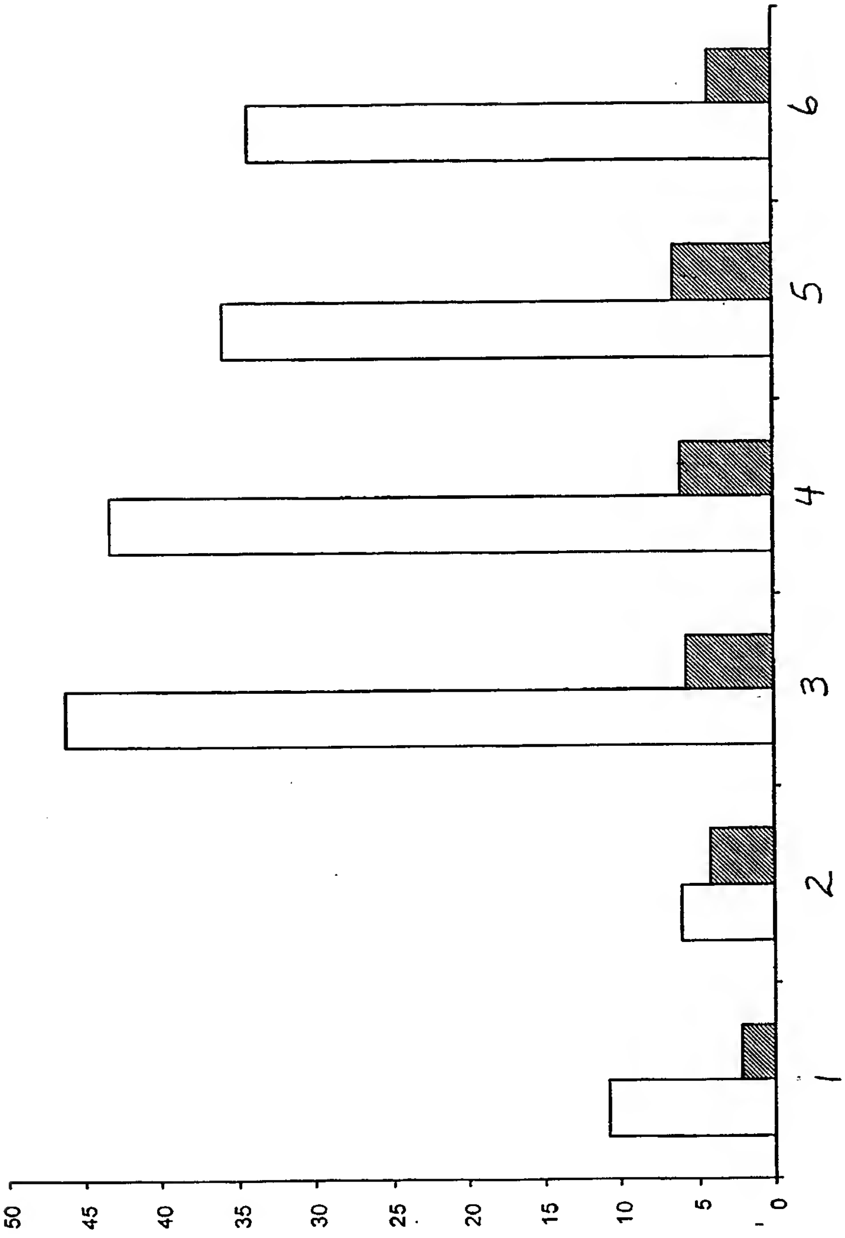
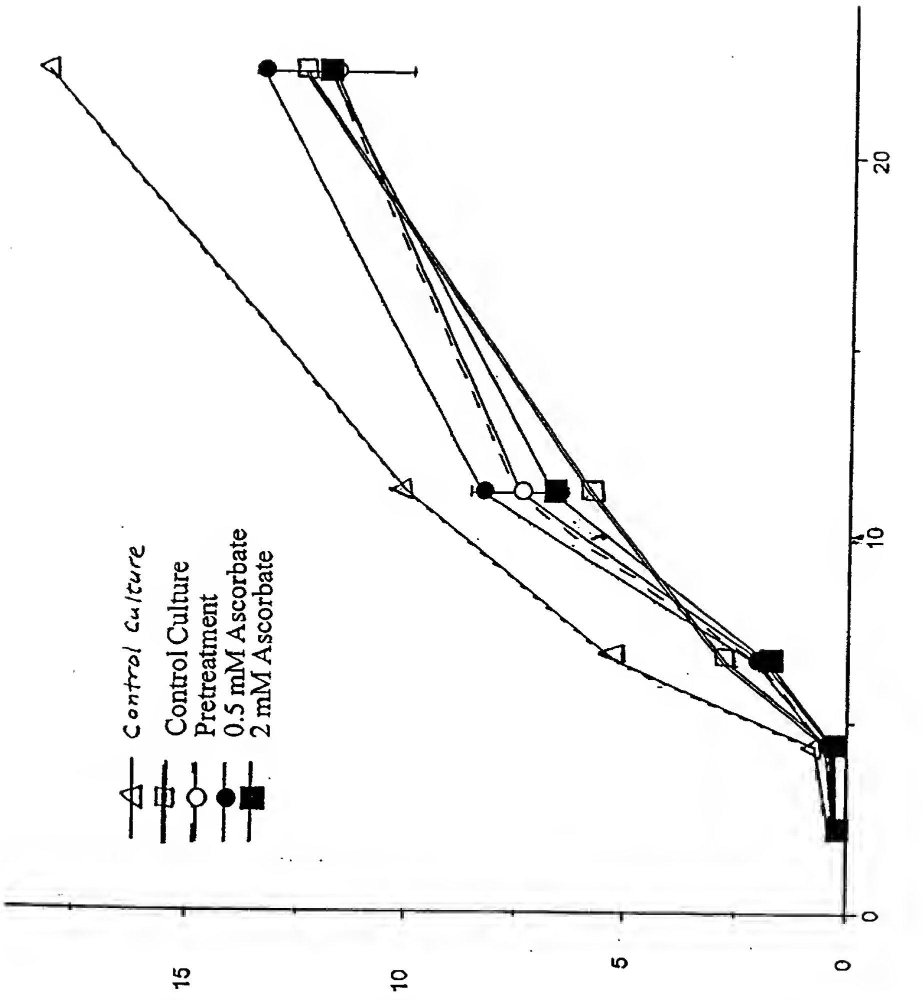


FIG. 25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Control Culture
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2 mM Ascorbate
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10
5
0
0
10
20

FIG. 26

SEQUENCE LISTING

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      CLARK, SAMUEL A.
      THIGPEN, ANICE E.
      KRUSE, FRED
      RHODES, CHRISTOPHER

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Arg Glu Glu Ser Leu Gln Leu Met Asp Leu Leu Gly Leu Glu Arg Ser
              10                  15                  20

gcc tgg ggg aat att cct ctg atg aga aag gca tat tta aaa aaa tgc   152
Ala Trp Gly Asn Ile Pro Leu Met Arg Lys Ala Tyr Leu Lys Lys Cys
          25                  30                  35

aag gag ttt cat cct gat aaa gga gga gat gaa gaa aaa atg aag aaa   200
Lys Glu Phe His Pro Asp Lys Gly Gly Asp Glu Glu Lys Met Lys Lys
      40                  45                  50

atg aat act ctg tac aag aaa atg gaa gat gga gta aaa tat gct cat   248
Met Asn Thr Leu Tyr Lys Lys Met Glu Asp Gly Val Lys Tyr Ala His
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Gln Pro Asp Phe Gly Gly Phe Trp Asp Ala Thr Glu
                 75                  80

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aaatgtctgc taactgcata tgcttgctgt gcttactgag gatgaagcat gaaaatagaa 414

aattatacag gaaagatcca cttgtgtggg ttgattgcta ctgcttcgat tgctttagaa 474

tgtggtttgg acttgatctt tgtgaaggaa ccttacttct gtggtgtgac ataattggac 534

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gttaaactac tgattctaat tgtttgtgta ttttag att cca acc tat gga act   648
                                        Ile Pro Thr Tyr Gly Thr
                                                    85

gat gaa tgg gag cag tgg tgg aat gcc ttt aat gag gaa aac ctg ttt   696
Asp Glu Trp Glu Gln Trp Trp Asn Ala Phe Asn Glu Glu Asn Leu Phe
     90                  95                 100

tgc tca gaa gaa atg cca tct agt gat gat gag gct act gct gac tct   744
Cys Ser Glu Glu Met Pro Ser Ser Asp Asp Glu Ala Thr Ala Asp Ser
105                 110                 115                 120

caa cat tct act cct cca aaa aag aag aga aag gta gaa gac ccc aag   792
Gln His Ser Thr Pro Pro Lys Lys Lys Arg Lys Val Glu Asp Pro Lys
                125                 130                 135

gac ttt cct tca gaa ttg cta agt ttt ttg agt cat gct gtg ttt agt   840
Asp Phe Pro Ser Glu Leu Leu Ser Phe Leu Ser His Ala Val Phe Ser
            140                 145                 150

aat aga act ctt gct tgc ttt gct att tac acc aca aag gaa aaa gct   888
Asn Arg Thr Leu Ala Cys Phe Ala Ile Tyr Thr Thr Lys Glu Lys Ala
        155                 160                 165

gca ctg cta tac aag aaa att atg gaa aaa tat tct gta acc ttt ata   936
Ala Leu Leu Tyr Lys Lys Ile Met Glu Lys Tyr Ser Val Thr Phe Ile
    170                 175                 180

agt agg cat aac agt tat aat cat aac ata ctg ttt ttt ctt act cca   984
Ser Arg His Asn Ser Tyr Asn His Asn Ile Leu Phe Phe Leu Thr Pro
185                 190                 195                 200

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His Arg His Arg Val Ser Ala Ile Asn Asn Tyr Ala Gln Lys Leu Cys
                205                 210                 215

acc ttt agc ttt tta att tgt aaa ggg gtt aat aag gaa tat ttg atg   1080
Thr Phe Ser Phe Leu Ile Cys Lys Gly Val Asn Lys Glu Tyr Leu Met
            220                 225                 230
```

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Tyr Ser Ala Leu Thr Arg Asp Pro Phe Ser Val Ile Glu Glu Ser Leu
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Pro Gly Gly Leu Lys Glu His Asp Phe Asn Pro Glu Glu Ala Glu Glu
    250                 255                 260

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Thr Lys Gln Val Ser Trp Lys Leu Val Thr Glu Tyr Ala Met Glu Thr
265                 270                 275                 280

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Lys Cys Asp Asp Val Leu Leu Leu Leu Gly Met Tyr Leu Glu Phe Gln
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            300                 305                 310

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His Tyr Lys Tyr His Glu Lys His Tyr Ala Asn Ala Ala Ile Phe Ala
        315                 320                 325

gac agc aaa aac caa aaa acc ata tgc caa cag gct gtt gat act gtt     1416
Asp Ser Lys Asn Gln Lys Thr Ile Cys Gln Gln Ala Val Asp Thr Val
    330                 335                 340

tta gct aaa aag cgg gtt gat agc cta caa tta act aga gaa caa atg     1464
Leu Ala Lys Lys Arg Val Asp Ser Leu Gln Leu Thr Arg Glu Gln Met
345                 350                 355                 360

tta aca aac aga ttt aat gat ctt ttg gat agg atg gat ata atg ttt     1512
Leu Thr Asn Arg Phe Asn Asp Leu Leu Asp Arg Met Asp Ile Met Phe
                365                 370                 375

ggt tct aca ggc tct gct gac ata gaa gaa tgg atg gct gga gtt gct     1560
Gly Ser Thr Gly Ser Ala Asp Ile Glu Glu Trp Met Ala Gly Val Ala
            380                 385                 390

tgg cta cac tgt ttg ttg ccc aaa atg gat tca gtg gtg tat gac ttt     1608
Trp Leu His Cys Leu Leu Pro Lys Met Asp Ser Val Val Tyr Asp Phe
        395                 400                 405

tta aaa tgc atg gtg tac aac att cct aaa aaa aga tac tgg ctg ttt     1656
Leu Lys Cys Met Val Tyr Asn Ile Pro Lys Lys Arg Tyr Trp Leu Phe
    410                 415                 420

aaa gga cca att gat agt ggt aaa act aca tta gca gct gct ttg ctt     1704
Lys Gly Pro Ile Asp Ser Gly Lys Thr Thr Leu Ala Ala Ala Leu Leu
425                 430                 435                 440

gaa tta tgt ggg ggg aaa gct tta aat gtt aat ttg ccc ttg gac agg     1752
Glu Leu Cys Gly Gly Lys Ala Leu Asn Val Asn Leu Pro Leu Asp Arg
                445                 450                 455
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ctg aac ttt gag cta gga gta gct att gac cag ttt tta gta gtt ttt    1800
Leu Asn Phe Glu Leu Gly Val Ala Ile Asp Gln Phe Leu Val Val Phe
            460                 465                 470

gag gat gta aag ggc act gga ggg gag tcc aga gat ttg cct tca ggt    1848
Glu Asp Val Lys Gly Thr Gly Gly Glu Ser Arg Asp Leu Pro Ser Gly
        475                 480                 485

cag gga att aat aac ctg gac aat tta agg gat tat ttg gat ggc agt    1896
Gln Gly Ile Asn Asn Leu Asp Asn Leu Arg Asp Tyr Leu Asp Gly Ser
    490                 495                 500

gtt aag gta aac tta gaa aag aaa cac cta aat aaa aga act caa ata    1944
Val Lys Val Asn Leu Glu Lys Lys His Leu Asn Lys Arg Thr Gln Ile
505                 510                 515                 520

ttt ccc cct gga ata gtc acc atg aat gag tac agt gtg cct aaa aca    1992
Phe Pro Pro Gly Ile Val Thr Met Asn Glu Tyr Ser Val Pro Lys Thr
                525                 530                 535

ctg cag gcc aga ttt gta aaa caa ata gat ttt agg ccc aaa gat tat    2040
Leu Gln Ala Arg Phe Val Lys Gln Ile Asp Phe Arg Pro Lys Asp Tyr
            540                 545                 550

tta aag cat tgc ctg gaa cgc agt gag ttt ttg tta gaa aag aga ata    2088
Leu Lys His Cys Leu Glu Arg Ser Glu Phe Leu Leu Glu Lys Arg Ile
        555                 560                 565

att caa agt ggc att gct ttg ctt ctt atg tta att tgg tac aga cct    2136
Ile Gln Ser Gly Ile Ala Leu Leu Leu Met Leu Ile Trp Tyr Arg Pro
    570                 575                 580

gtg gct gag ttt gct caa agt att cag agc aga att gtg gag tgg aaa    2184
Val Ala Glu Phe Ala Gln Ser Ile Gln Ser Arg Ile Val Glu Trp Lys
585                 590                 595                 600

gag aga ttg gac aaa gag ttt agt ttg tca gtg tat caa aaa atg aag    2232
Glu Arg Leu Asp Lys Glu Phe Ser Leu Ser Val Tyr Gln Lys Met Lys
                605                 610                 615

ttt aat gtg gct atg gga att gga gtt tta gat tgg cta aga aac agt    2280
Phe Asn Val Ala Met Gly Ile Gly Val Leu Asp Trp Leu Arg Asn Ser
            620                 625                 630

gat gat gat gat gaa gac agc cag gaa aat gct gat aaa aat gaa gat    2328
Asp Asp Asp Asp Glu Asp Ser Gln Glu Asn Ala Asp Lys Asn Glu Asp
        635                 640                 645

ggt ggg gag aag aac atg gaa gac tca ggg cat gaa aca ggc att gat    2376
Gly Gly Glu Lys Asn Met Glu Asp Ser Gly His Glu Thr Gly Ile Asp
    650                 655                 660

tca cag tcc caa ggc tca ttt cag gcc cct cag tcc tca cag tct gtt    2424
Ser Gln Ser Gln Gly Ser Phe Gln Ala Pro Gln Ser Ser Gln Ser Val
665                 670                 675                 680
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cat gat cat aat cag cca tac cac att tgt aga ggt ttt act tgc ttt      2472
His Asp His Asn Gln Pro Tyr His Ile Cys Arg Gly Phe Thr Cys Phe
            685                 690                 695

aaa aaa cct ccc aca cct ccc cct gaa cct gaa aca taaaatgaat           2518
Lys Lys Pro Pro Thr Pro Pro Pro Glu Pro Glu Thr
        700                 705

gcaattgttg ttgtt                                                     2533


<210> 2
<211> 708
<212> PRT
<213> simian virus 40

<400> 2
Met Asp Lys Val Leu Asn Arg Glu Glu Ser Leu Gln Leu Met Asp Leu
 1               5                  10                  15

Leu Gly Leu Glu Arg Ser Ala Trp Gly Asn Ile Pro Leu Met Arg Lys
            20                  25                  30

Ala Tyr Leu Lys Lys Cys Lys Glu Phe His Pro Asp Lys Gly Gly Asp
        35                  40                  45

Glu Glu Lys Met Lys Lys Met Asn Thr Leu Tyr Lys Lys Met Glu Asp
    50                  55                  60

Gly Val Lys Tyr Ala His Gln Pro Asp Phe Gly Gly Phe Trp Asp Ala
65                  70                  75                  80

Thr Glu Ile Pro Thr Tyr Gly Thr Asp Glu Trp Glu Gln Trp Trp Asn
                85                  90                  95

Ala Phe Asn Glu Glu Asn Leu Phe Cys Ser Glu Glu Met Pro Ser Ser
            100                 105                 110

Asp Asp Glu Ala Thr Ala Asp Ser Gln His Ser Thr Pro Pro Lys Lys
        115                 120                 125

Lys Arg Lys Val Glu Asp Pro Lys Asp Phe Pro Ser Glu Leu Leu Ser
    130                 135                 140

Phe Leu Ser His Ala Val Phe Ser Asn Arg Thr Leu Ala Cys Phe Ala
145                 150                 155                 160

Ile Tyr Thr Thr Lys Glu Lys Ala Ala Leu Leu Tyr Lys Lys Ile Met
                165                 170                 175

Glu Lys Tyr Ser Val Thr Phe Ile Ser Arg His Asn Ser Tyr Asn His
            180                 185                 190

Asn Ile Leu Phe Phe Leu Thr Pro His Arg His Arg Val Ser Ala Ile
        195                 200                 205
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Asn Asn Tyr Ala Gln Lys Leu Cys Thr Phe Ser Phe Leu Ile Cys Lys
    210                 215                 220

Gly Val Asn Lys Glu Tyr Leu Met Tyr Ser Ala Leu Thr Arg Asp Pro
225                 230                 235                 240

Phe Ser Val Ile Glu Glu Ser Leu Pro Gly Gly Leu Lys Glu His Asp
                245                 250                 255

Phe Asn Pro Glu Glu Ala Glu Glu Thr Lys Gln Val Ser Trp Lys Leu
            260                 265                 270

Val Thr Glu Tyr Ala Met Glu Thr Lys Cys Asp Asp Val Leu Leu Leu
        275                 280                 285

Leu Gly Met Tyr Leu Glu Phe Gln Tyr Ser Phe Glu Met Cys Leu Lys
    290                 295                 300

Cys Ile Lys Lys Glu Gln Pro Ser His Tyr Lys Tyr His Glu Lys His
305                 310                 315                 320

Tyr Ala Asn Ala Ala Ile Phe Ala Asp Ser Lys Asn Gln Lys Thr Ile
                325                 330                 335

Cys Gln Gln Ala Val Asp Thr Val Leu Ala Lys Lys Arg Val Asp Ser
            340                 345                 350

Leu Gln Leu Thr Arg Glu Gln Met Leu Thr Asn Arg Phe Asn Asp Leu
        355                 360                 365

Leu Asp Arg Met Asp Ile Met Phe Gly Ser Thr Gly Ser Ala Asp Ile
    370                 375                 380

Glu Glu Trp Met Ala Gly Val Ala Trp Leu His Cys Leu Leu Pro Lys
385                 390                 395                 400

Met Asp Ser Val Val Tyr Asp Phe Leu Lys Cys Met Val Tyr Asn Ile
                405                 410                 415

Pro Lys Lys Arg Tyr Trp Leu Phe Lys Gly Pro Ile Asp Ser Gly Lys
            420                 425                 430

Thr Thr Leu Ala Ala Ala Leu Leu Glu Leu Cys Gly Gly Lys Ala Leu
        435                 440                 445

Asn Val Asn Leu Pro Leu Asp Arg Leu Asn Phe Glu Leu Gly Val Ala
    450                 455                 460

Ile Asp Gln Phe Leu Val Val Phe Glu Asp Val Lys Gly Thr Gly Gly
465                 470                 475                 480

Glu Ser Arg Asp Leu Pro Ser Gly Gln Gly Ile Asn Asn Leu Asp Asn
                485                 490                 495

Leu Arg Asp Tyr Leu Asp Gly Ser Val Lys Val Asn Leu Glu Lys Lys
            500                 505                 510
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His Leu Asn Lys Arg Thr Gln Ile Phe Pro Pro Gly Ile Val Thr Met
515 520 525

Asn Glu Tyr Ser Val Pro Lys Thr Leu Gln Ala Arg Phe Val Lys Gln
530 535 540

Ile Asp Phe Arg Pro Lys Asp Tyr Leu Lys His Cys Leu Glu Arg Ser
545 550 555 560

Glu Phe Leu Leu Glu Lys Arg Ile Ile Gln Ser Gly Ile Ala Leu Leu
565 570 575

Leu Met Leu Ile Trp Tyr Arg Pro Val Ala Glu Phe Ala Gln Ser Ile
580 585 590

Gln Ser Arg Ile Val Glu Trp Lys Glu Arg Leu Asp Lys Glu Phe Ser
595 600 605

Leu Ser Val Tyr Gln Lys Met Lys Phe Asn Val Ala Met Gly Ile Gly
610 615 620

Val Leu Asp Trp Leu Arg Asn Ser Asp Asp Asp Asp Glu Asp Ser Gln
625 630 635 640

Glu Asn Ala Asp Lys Asn Glu Asp Gly Gly Glu Lys Asn Met Glu Asp
645 650 655

Ser Gly His Glu Thr Gly Ile Asp Ser Gln Ser Gln Gly Ser Phe Gln
660 665 670

Ala Pro Gln Ser Ser Gln Ser Val His Asp His Asn Gln Pro Tyr His
675 680 685

Ile Cys Arg Gly Phe Thr Cys Phe Lys Lys Pro Pro Thr Pro Pro Pro
690 695 700

Glu Pro Glu Thr
705

<210> 3
<211> 82
<212> PRT
<213> simian virus 40

<400> 3
Met Asp Lys Val Leu Asn Arg Glu Glu Ser Leu Gln Leu Met Asp Leu
1 5 10 15

Leu Gly Leu Glu Arg Ser Ala Trp Gly Asn Ile Pro Leu Met Arg Lys
20 25 30

Ala Tyr Leu Lys Lys Cys Lys Glu Phe His Pro Asp Lys Gly Gly Asp
35 40 45

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Glu Glu Lys Met Lys Lys Met Asn Thr Leu Tyr Lys Lys Met Glu Asp
     50                  55                  60

Gly Val Lys Tyr Ala His Gln Pro Asp Phe Gly Gly Phe Trp Asp Ala
 65                  70                  75                  80

Thr Glu


<210> 4
<211> 626
<212> PRT
<213> simian virus 40

<400> 4
Ile Pro Thr Tyr Gly Thr Asp Glu Trp Glu Gln Trp Trp Asn Ala Phe
  1               5                  10                  15

Asn Glu Glu Asn Leu Phe Cys Ser Glu Glu Met Pro Ser Ser Asp Asp
             20                  25                  30

Glu Ala Thr Ala Asp Ser Gln His Ser Thr Pro Pro Lys Lys Lys Arg
         35                  40                  45

Lys Val Glu Asp Pro Lys Asp Phe Pro Ser Glu Leu Leu Ser Phe Leu
     50                  55                  60

Ser His Ala Val Phe Ser Asn Arg Thr Leu Ala Cys Phe Ala Ile Tyr
 65                  70                  75                  80

Thr Thr Lys Glu Lys Ala Ala Leu Leu Tyr Lys Lys Ile Met Glu Lys
                 85                  90                  95

Tyr Ser Val Thr Phe Ile Ser Arg His Asn Ser Tyr Asn His Asn Ile
            100                 105                 110

Leu Phe Phe Leu Thr Pro His Arg His Arg Val Ser Ala Ile Asn Asn
        115                 120                 125

Tyr Ala Gln Lys Leu Cys Thr Phe Ser Phe Leu Ile Cys Lys Gly Val
    130                 135                 140

Asn Lys Glu Tyr Leu Met Tyr Ser Ala Leu Thr Arg Asp Pro Phe Ser
145                 150                 155                 160

Val Ile Glu Glu Ser Leu Pro Gly Gly Leu Lys Glu His Asp Phe Asn
                165                 170                 175

Pro Glu Glu Ala Glu Glu Thr Lys Gln Val Ser Trp Lys Leu Val Thr
            180                 185                 190

Glu Tyr Ala Met Glu Thr Lys Cys Asp Asp Val Leu Leu Leu Leu Gly
        195                 200                 205

Met Tyr Leu Glu Phe Gln Tyr Ser Phe Glu Met Cys Leu Lys Cys Ile
    210                 215                 220
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Lys Lys Glu Gln Pro Ser His Tyr Lys Tyr His Glu Lys His Tyr Ala
225                 230                 235                 240

Asn Ala Ala Ile Phe Ala Asp Ser Lys Asn Gln Lys Thr Ile Cys Gln
                245                 250                 255

Gln Ala Val Asp Thr Val Leu Ala Lys Lys Arg Val Asp Ser Leu Gln
            260                 265                 270

Leu Thr Arg Glu Gln Met Leu Thr Asn Arg Phe Asn Asp Leu Leu Asp
        275                 280                 285

Arg Met Asp Ile Met Phe Gly Ser Thr Gly Ser Ala Asp Ile Glu Glu
    290                 295                 300

Trp Met Ala Gly Val Ala Trp Leu His Cys Leu Leu Pro Lys Met Asp
305                 310                 315                 320

Ser Val Val Tyr Asp Phe Leu Lys Cys Met Val Tyr Asn Ile Pro Lys
                325                 330                 335

Lys Arg Tyr Trp Leu Phe Lys Gly Pro Ile Asp Ser Gly Lys Thr Thr
            340                 345                 350

Leu Ala Ala Ala Leu Leu Glu Leu Cys Gly Gly Lys Ala Leu Asn Val
        355                 360                 365

Asn Leu Pro Leu Asp Arg Leu Asn Phe Glu Leu Gly Val Ala Ile Asp
    370                 375                 380

Gln Phe Leu Val Val Phe Glu Asp Val Lys Gly Thr Gly Gly Glu Ser
385                 390                 395                 400

Arg Asp Leu Pro Ser Gly Gln Gly Ile Asn Asn Leu Asp Asn Leu Arg
                405                 410                 415

Asp Tyr Leu Asp Gly Ser Val Lys Val Asn Leu Glu Lys Lys His Leu
            420                 425                 430

Asn Lys Arg Thr Gln Ile Phe Pro Pro Gly Ile Val Thr Met Asn Glu
        435                 440                 445

Tyr Ser Val Pro Lys Thr Leu Gln Ala Arg Phe Val Lys Gln Ile Asp
    450                 455                 460

Phe Arg Pro Lys Asp Tyr Leu Lys His Cys Leu Glu Arg Ser Glu Phe
465                 470                 475                 480

Leu Leu Glu Lys Arg Ile Ile Gln Ser Gly Ile Ala Leu Leu Leu Met
                485                 490                 495

Leu Ile Trp Tyr Arg Pro Val Ala Glu Phe Ala Gln Ser Ile Gln Ser
            500                 505                 510

Arg Ile Val Glu Trp Lys Glu Arg Leu Asp Lys Glu Phe Ser Leu Ser
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        515                 520                 525

Val Tyr Gln Lys Met Lys Phe Asn Val Ala Met Gly Ile Gly Val Leu
    530                 535                 540

Asp Trp Leu Arg Asn Ser Asp Asp Asp Asp Glu Asp Ser Gln Glu Asn
545                 550                 555                 560

Ala Asp Lys Asn Glu Asp Gly Gly Glu Lys Asn Met Glu Asp Ser Gly
                565                 570                 575

His Glu Thr Gly Ile Asp Ser Gln Ser Gln Gly Ser Phe Gln Ala Pro
            580                 585                 590

Gln Ser Ser Gln Ser Val His Asp His Asn Gln Pro Tyr His Ile Cys
        595                 600                 605

Arg Gly Phe Thr Cys Phe Lys Lys Pro Pro Thr Pro Pro Pro Glu Pro
    610                 615                 620

Glu Thr
625


<210> 5
<211> 2533
<212> DNA
<213> simian virus 40

<220>
<221> CDS
<222> (39)..(284)

<220>
<221> CDS
<222> (631)..(2508)

<400> 5
gaattcgatc ctaggctttt gcaaaagct ttgcaaag atg gat aaa gtt tta aac 56
                                          Met Asp Lys Val Leu Asn
                                            1               5

aga gag gaa tct ttg cag cta atg gac ctt cta ggt ctt gaa agg agt    104
Arg Glu Glu Ser Leu Gln Leu Met Asp Leu Leu Gly Leu Glu Arg Ser
             10                  15                  20

gcc tgg ggg aat att cct ctg atg aga aag gca tat tta aaa aaa tgc    152
Ala Trp Gly Asn Ile Pro Leu Met Arg Lys Ala Tyr Leu Lys Lys Cys
         25                  30                  35

aag gag ttt cat cct gat aaa gga gga gat gaa gaa aaa atg aag aaa    200
Lys Glu Phe His Pro Asp Lys Gly Gly Asp Glu Glu Lys Met Lys Lys
     40                  45                  50

atg aat act ctg tac aag aaa atg gaa gat gga gta aaa tat gct cat    248
Met Asn Thr Leu Tyr Lys Lys Met Glu Asp Gly Val Lys Tyr Ala His
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 55                  60                  65                  70

caa cct gac ttt gga ggc ttc tgg gat gca act gag gtatttgctt        294
Gln Pro Asp Phe Gly Gly Phe Trp Asp Ala Thr Glu
                75                  80

cttccttaaa tcctggtgtt gatgcaatgt actgcaaaca atggcctgag tgtgcaaaga 354

aaatgtctgc taactgcata tgcttgctgt gcttactgag gatgaagcat gaaaatagaa 414

aattatacag gaaagatcca cttgtgtggg ttgattgcta ctgcttcgat tgctttagaa 474

tgtggtttgg acttgatctt tgtgaaggaa ccttacttct gtggtgtgac ataattggac 534

aaactaccta cagagattta aagctctaag gtaaatataa aatttttaag tgtataatgt 594

gttaaactac tgattctaat tgtttgtgta ttttag att cca acc tat gga act   648
                                        Ile Pro Thr Tyr Gly Thr
                                                     85

gat gaa tgg gag cag tgg tgg aat gcc ttt aat gag gaa aac ctg ttt   696
Asp Glu Trp Glu Gln Trp Trp Asn Ala Phe Asn Glu Glu Asn Leu Phe
    90                  95                  100

tgc tca gaa gaa atg cca tct agt gat gat gag gct act gct gac tct   744
Cys Ser Glu Glu Met Pro Ser Ser Asp Asp Glu Ala Thr Ala Asp Ser
105                 110                 115                 120

caa cat tct act cct cca aaa aag aag aga aag gta gaa gac ccc aag   792
Gln His Ser Thr Pro Pro Lys Lys Lys Arg Lys Val Glu Asp Pro Lys
                125                 130                 135

gac ttt cct tca gaa ttg cta agt ttt ttg agt cat gct gtg ttt agt   840
Asp Phe Pro Ser Glu Leu Leu Ser Phe Leu Ser His Ala Val Phe Ser
            140                 145                 150

aat aga act ctt gct tgc ttt gct att tac acc aca aag gaa aaa gct   888
Asn Arg Thr Leu Ala Cys Phe Ala Ile Tyr Thr Thr Lys Glu Lys Ala
        155                 160                 165

gca ctg cta tac aag aaa att atg gaa aaa tat tct gta acc ttt ata   936
Ala Leu Leu Tyr Lys Lys Ile Met Glu Lys Tyr Ser Val Thr Phe Ile
    170                 175                 180

agt agg cat aac agt tat aat cat aac ata ctg ttt ttt ctt act cca   984
Ser Arg His Asn Ser Tyr Asn His Asn Ile Leu Phe Phe Leu Thr Pro
185                 190                 195                 200

cac agg cat aga gtg tct gct att aat aac tat gct caa aaa ttg tgt   1032
His Arg His Arg Val Ser Ala Ile Asn Asn Tyr Ala Gln Lys Leu Cys
                205                 210                 215

acc ttt agc ttt tta att tgt aaa ggg gtt aat aag gaa tat ttg atg   1080
Thr Phe Ser Phe Leu Ile Cys Lys Gly Val Asn Lys Glu Tyr Leu Met
            220                 225                 230
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tat agt gcc ttg act aga gat cca ttt tct gtt att gag gaa agt ttg    1128
Tyr Ser Ala Leu Thr Arg Asp Pro Phe Ser Val Ile Glu Glu Ser Leu
        235                 240                 245

cca ggt ggg tta aag gag cat gat ttt aat cca gaa gaa gca gag gaa    1176
Pro Gly Gly Leu Lys Glu His Asp Phe Asn Pro Glu Glu Ala Glu Glu
    250                 255                 260

act aaa caa gtg tcc tgg aag ctt gta aca gag tat gca atg gaa aca    1224
Thr Lys Gln Val Ser Trp Lys Leu Val Thr Glu Tyr Ala Met Glu Thr
265                 270                 275                 280

aaa tgt gat gat gtg ttg tta ttg ctt ggg atg tac ttg gaa ttt cag    1272
Lys Cys Asp Asp Val Leu Leu Leu Leu Gly Met Tyr Leu Glu Phe Gln
                285                 290                 295

tac agt ttt gaa atg tgt tta aaa tgt att aaa aaa gaa cag ccc agc    1320
Tyr Ser Phe Glu Met Cys Leu Lys Cys Ile Lys Lys Glu Gln Pro Ser
            300                 305                 310

cac tat aag tac cat gaa aag cat tat gca aat gct gct ata ttt gct    1368
His Tyr Lys Tyr His Glu Lys His Tyr Ala Asn Ala Ala Ile Phe Ala
        315                 320                 325

gac agc aaa aac caa aaa acc ata tgc caa cag gct gtt gat act gtt    1416
Asp Ser Lys Asn Gln Lys Thr Ile Cys Gln Gln Ala Val Asp Thr Val
    330                 335                 340

tta gct aaa aag cgg gtt gat agc cta caa tta act aga gaa caa atg    1464
Leu Ala Lys Lys Arg Val Asp Ser Leu Gln Leu Thr Arg Glu Gln Met
345                 350                 355                 360

tta aca aac aga ttt aat gat ctt ttg gat agg atg gat ata atg ttt    1512
Leu Thr Asn Arg Phe Asn Asp Leu Leu Asp Arg Met Asp Ile Met Phe
                365                 370                 375

ggt tct aca ggc tct gct gac ata gaa gaa tgg atg gct gga gtt gct    1560
Gly Ser Thr Gly Ser Ala Asp Ile Glu Glu Trp Met Ala Gly Val Ala
            380                 385                 390

tgg cta cac tgt ttg ttg ccc aaa atg gat tca gtg gtg tat gac ttt    1608
Trp Leu His Cys Leu Leu Pro Lys Met Asp Ser Val Val Tyr Asp Phe
        395                 400                 405

tta aaa tgc atg gtg tac aac att cct aaa aaa aga tac tgg ctg ttt    1656
Leu Lys Cys Met Val Tyr Asn Ile Pro Lys Lys Arg Tyr Trp Leu Phe
    410                 415                 420

aaa gga cca att gat agt ggt aaa act aca tta gca gct gtt ttg ctt    1704
Lys Gly Pro Ile Asp Ser Gly Lys Thr Thr Leu Ala Ala Val Leu Leu
425                 430                 435                 440

gaa tta tgt ggg ggg aaa gct tta aat gtt aat ttg ccc ttg gac agg    1752
Glu Leu Cys Gly Gly Lys Ala Leu Asn Val Asn Leu Pro Leu Asp Arg
                445                 450                 455
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ctg aac ttt gag cta gga gta gct att gac cag ttt tta gta gtt ttt    1800
Leu Asn Phe Glu Leu Gly Val Ala Ile Asp Gln Phe Leu Val Val Phe
            460                 465                 470

gag gat gta aag ggc act gga ggg gag tcc aga gat ttg cct tca ggt    1848
Glu Asp Val Lys Gly Thr Gly Gly Glu Ser Arg Asp Leu Pro Ser Gly
        475                 480                 485

cag gga att aat aac ctg gac aat tta agg gat tat ttg gat ggc agt    1896
Gln Gly Ile Asn Asn Leu Asp Asn Leu Arg Asp Tyr Leu Asp Gly Ser
    490                 495                 500

gtt aag gta aac tta gaa aag aaa cac cta aat aaa aga act caa ata    1944
Val Lys Val Asn Leu Glu Lys Lys His Leu Asn Lys Arg Thr Gln Ile
505                 510                 515                 520

ttt ccc cct gga ata gtc acc atg aat gag tac agt gtg cct aaa aca    1992
Phe Pro Pro Gly Ile Val Thr Met Asn Glu Tyr Ser Val Pro Lys Thr
                525                 530                 535

ctg cag gcc aga ttt gta aaa caa ata gat ttt agg ccc aaa gat tat    2040
Leu Gln Ala Arg Phe Val Lys Gln Ile Asp Phe Arg Pro Lys Asp Tyr
            540                 545                 550

tta aag cat tgc ctg gaa cgc agt gag ttt ttg tta gaa aag aga ata    2088
Leu Lys His Cys Leu Glu Arg Ser Glu Phe Leu Leu Glu Lys Arg Ile
        555                 560                 565

att caa agt ggc att gct ttg ctt ctt atg tta att tgg tac aga cct    2136
Ile Gln Ser Gly Ile Ala Leu Leu Leu Met Leu Ile Trp Tyr Arg Pro
    570                 575                 580

gtg gct gag ttt gct caa agt att cag agc aga att gtg gag tgg aaa    2184
Val Ala Glu Phe Ala Gln Ser Ile Gln Ser Arg Ile Val Glu Trp Lys
585                 590                 595                 600

gag aga ttg gac aaa gag ttt agt ttg tca gtg tat caa aaa atg aag    2232
Glu Arg Leu Asp Lys Glu Phe Ser Leu Ser Val Tyr Gln Lys Met Lys
                605                 610                 615

ttt aat gtg gct atg gga att gga gtt tta gat tgg cta aga aac agt    2280
Phe Asn Val Ala Met Gly Ile Gly Val Leu Asp Trp Leu Arg Asn Ser
            620                 625                 630

gat gat gat gat gaa gac agc cag gaa aat gct gat aaa aat gaa gat    2328
Asp Asp Asp Asp Glu Asp Ser Gln Glu Asn Ala Asp Lys Asn Glu Asp
        635                 640                 645

ggt ggg gag aag aac atg gaa gac tca ggg cat gaa aca ggc att gat    2376
Gly Gly Glu Lys Asn Met Glu Asp Ser Gly His Glu Thr Gly Ile Asp
    650                 655                 660

tca cag tcc caa ggc tca ttt cag gcc cct cag tcc tca cag tct gtt    2424
Ser Gln Ser Gln Gly Ser Phe Gln Ala Pro Gln Ser Ser Gln Ser Val
665                 670                 675                 680
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cat gat cat aat cag cca tac cac att tgt aga ggt ttt act tgc ttt     2472
His Asp His Asn Gln Pro Tyr His Ile Cys Arg Gly Phe Thr Cys Phe
                685                 690                 695

aaa aaa cct ccc aca cct ccc cct gaa cct gaa aca taaaatgaat          2518
Lys Lys Pro Pro Thr Pro Pro Pro Glu Pro Glu Thr
            700                 705

gcaattgttg ttgtt                                                    2533


<210> 6
<211> 708
<212> PRT
<213> simian virus 40

<400> 6
Met Asp Lys Val Leu Asn Arg Glu Glu Ser Leu Gln Leu Met Asp Leu
 1               5                  10                  15

Leu Gly Leu Glu Arg Ser Ala Trp Gly Asn Ile Pro Leu Met Arg Lys
             20                  25                  30

Ala Tyr Leu Lys Lys Cys Lys Glu Phe His Pro Asp Lys Gly Gly Asp
         35                  40                  45

Glu Glu Lys Met Lys Lys Met Asn Thr Leu Tyr Lys Lys Met Glu Asp
     50                  55                  60

Gly Val Lys Tyr Ala His Gln Pro Asp Phe Gly Gly Phe Trp Asp Ala
 65                  70                  75                  80

Thr Glu Ile Pro Thr Tyr Gly Thr Asp Glu Trp Glu Gln Trp Trp Asn
                 85                  90                  95

Ala Phe Asn Glu Glu Asn Leu Phe Cys Ser Glu Glu Met Pro Ser Ser
            100                 105                 110

Asp Asp Glu Ala Thr Ala Asp Ser Gln His Ser Thr Pro Pro Lys Lys
        115                 120                 125

Lys Arg Lys Val Glu Asp Pro Lys Asp Phe Pro Ser Glu Leu Leu Ser
    130                 135                 140

Phe Leu Ser His Ala Val Phe Ser Asn Arg Thr Leu Ala Cys Phe Ala
145                 150                 155                 160

Ile Tyr Thr Thr Lys Glu Lys Ala Ala Leu Leu Tyr Lys Lys Ile Met
                165                 170                 175

Glu Lys Tyr Ser Val Thr Phe Ile Ser Arg His Asn Ser Tyr Asn His
            180                 185                 190

Asn Ile Leu Phe Phe Leu Thr Pro His Arg His Arg Val Ser Ala Ile
        195                 200                 205
```

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Asn Asn Tyr Ala Gln Lys Leu Cys Thr Phe Ser Phe Leu Ile Cys Lys
    210                 215                 220

Gly Val Asn Lys Glu Tyr Leu Met Tyr Ser Ala Leu Thr Arg Asp Pro
225                 230                 235                 240

Phe Ser Val Ile Glu Glu Ser Leu Pro Gly Gly Leu Lys Glu His Asp
                245                 250                 255

Phe Asn Pro Glu Glu Ala Glu Glu Thr Lys Gln Val Ser Trp Lys Leu
            260                 265                 270

Val Thr Glu Tyr Ala Met Glu Thr Lys Cys Asp Asp Val Leu Leu Leu
        275                 280                 285

Leu Gly Met Tyr Leu Glu Phe Gln Tyr Ser Phe Glu Met Cys Leu Lys
    290                 295                 300

Cys Ile Lys Lys Glu Gln Pro Ser His Tyr Lys Tyr His Glu Lys His
305                 310                 315                 320

Tyr Ala Asn Ala Ala Ile Phe Ala Asp Ser Lys Asn Gln Lys Thr Ile
                325                 330                 335

Cys Gln Gln Ala Val Asp Thr Val Leu Ala Lys Lys Arg Val Asp Ser
            340                 345                 350

Leu Gln Leu Thr Arg Glu Gln Met Leu Thr Asn Arg Phe Asn Asp Leu
        355                 360                 365

Leu Asp Arg Met Asp Ile Met Phe Gly Ser Thr Gly Ser Ala Asp Ile
    370                 375                 380

Glu Glu Trp Met Ala Gly Val Ala Trp Leu His Cys Leu Leu Pro Lys
385                 390                 395                 400

Met Asp Ser Val Val Tyr Asp Phe Leu Lys Cys Met Val Tyr Asn Ile
                405                 410                 415

Pro Lys Lys Arg Tyr Trp Leu Phe Lys Gly Pro Ile Asp Ser Gly Lys
            420                 425                 430

Thr Thr Leu Ala Ala Val Leu Leu Glu Leu Cys Gly Gly Lys Ala Leu
        435                 440                 445

Asn Val Asn Leu Pro Leu Asp Arg Leu Asn Phe Glu Leu Gly Val Ala
    450                 455                 460

Ile Asp Gln Phe Leu Val Val Phe Glu Asp Val Lys Gly Thr Gly Gly
465                 470                 475                 480

Glu Ser Arg Asp Leu Pro Ser Gly Gln Gly Ile Asn Asn Leu Asp Asn
                485                 490                 495

Leu Arg Asp Tyr Leu Asp Gly Ser Val Lys Val Asn Leu Glu Lys Lys
            500                 505                 510
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His Leu Asn Lys Arg Thr Gln Ile Phe Pro Pro Gly Ile Val Thr Met
        515                 520                 525

Asn Glu Tyr Ser Val Pro Lys Thr Leu Gln Ala Arg Phe Val Lys Gln
    530                 535                 540

Ile Asp Phe Arg Pro Lys Asp Tyr Leu Lys His Cys Leu Glu Arg Ser
545                 550                 555                 560

Glu Phe Leu Leu Glu Lys Arg Ile Ile Gln Ser Gly Ile Ala Leu Leu
                565                 570                 575

Leu Met Leu Ile Trp Tyr Arg Pro Val Ala Glu Phe Ala Gln Ser Ile
            580                 585                 590

Gln Ser Arg Ile Val Glu Trp Lys Glu Arg Leu Asp Lys Glu Phe Ser
        595                 600                 605

Leu Ser Val Tyr Gln Lys Met Lys Phe Asn Val Ala Met Gly Ile Gly
    610                 615                 620

Val Leu Asp Trp Leu Arg Asn Ser Asp Asp Asp Asp Glu Asp Ser Gln
625                 630                 635                 640

Glu Asn Ala Asp Lys Asn Glu Asp Gly Gly Glu Lys Asn Met Glu Asp
                645                 650                 655

Ser Gly His Glu Thr Gly Ile Asp Ser Gln Ser Gln Gly Ser Phe Gln
            660                 665                 670

Ala Pro Gln Ser Ser Gln Ser Val His Asp His Asn Gln Pro Tyr His
        675                 680                 685

Ile Cys Arg Gly Phe Thr Cys Phe Lys Lys Pro Pro Thr Pro Pro Pro
    690                 695                 700

Glu Pro Glu Thr
705


<210> 7
<211> 82
<212> PRT
<213> simian virus 40

<400> 7
Met Asp Lys Val Leu Asn Arg Glu Glu Ser Leu Gln Leu Met Asp Leu
  1               5                   10                  15

Leu Gly Leu Glu Arg Ser Ala Trp Gly Asn Ile Pro Leu Met Arg Lys
             20                  25                  30

Ala Tyr Leu Lys Lys Cys Lys Glu Phe His Pro Asp Lys Gly Gly Asp
         35                  40                  45
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```
Glu Glu Lys Met Lys Lys Met Asn Thr Leu Tyr Lys Lys Met Glu Asp
    50                  55                  60

Gly Val Lys Tyr Ala His Gln Pro Asp Phe Gly Gly Phe Trp Asp Ala
65                  70                  75                  80

Thr Glu


<210> 8
<211> 626
<212> PRT
<213> simian virus 40

<400> 8
Ile Pro Thr Tyr Gly Thr Asp Glu Trp Glu Gln Trp Trp Asn Ala Phe
1               5                   10                  15

Asn Glu Glu Asn Leu Phe Cys Ser Glu Glu Met Pro Ser Ser Asp Asp
            20                  25                  30

Glu Ala Thr Ala Asp Ser Gln His Ser Thr Pro Pro Lys Lys Lys Arg
        35                  40                  45

Lys Val Glu Asp Pro Lys Asp Phe Pro Ser Glu Leu Leu Ser Phe Leu
    50                  55                  60

Ser His Ala Val Phe Ser Asn Arg Thr Leu Ala Cys Phe Ala Ile Tyr
65                  70                  75                  80

Thr Thr Lys Glu Lys Ala Ala Leu Leu Tyr Lys Lys Ile Met Glu Lys
                85                  90                  95

Tyr Ser Val Thr Phe Ile Ser Arg His Asn Ser Tyr Asn His Asn Ile
            100                 105                 110

Leu Phe Phe Leu Thr Pro His Arg His Arg Val Ser Ala Ile Asn Asn
        115                 120                 125

Tyr Ala Gln Lys Leu Cys Thr Phe Ser Phe Leu Ile Cys Lys Gly Val
    130                 135                 140

Asn Lys Glu Tyr Leu Met Tyr Ser Ala Leu Thr Arg Asp Pro Phe Ser
145                 150                 155                 160

Val Ile Glu Glu Ser Leu Pro Gly Gly Leu Lys Glu His Asp Phe Asn
                165                 170                 175

Pro Glu Glu Ala Glu Glu Thr Lys Gln Val Ser Trp Lys Leu Val Thr
            180                 185                 190

Glu Tyr Ala Met Glu Thr Lys Cys Asp Asp Val Leu Leu Leu Leu Gly
        195                 200                 205

Met Tyr Leu Glu Phe Gln Tyr Ser Phe Glu Met Cys Leu Lys Cys Ile
    210                 215                 220
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```
Lys Lys Glu Gln Pro Ser His Tyr Lys Tyr His Glu Lys His Tyr Ala
225                 230                 235                 240

Asn Ala Ala Ile Phe Ala Asp Ser Lys Asn Gln Lys Thr Ile Cys Gln
                245                 250                 255

Gln Ala Val Asp Thr Val Leu Ala Lys Lys Arg Val Asp Ser Leu Gln
            260                 265                 270

Leu Thr Arg Glu Gln Met Leu Thr Asn Arg Phe Asn Asp Leu Leu Asp
        275                 280                 285

Arg Met Asp Ile Met Phe Gly Ser Thr Gly Ser Ala Asp Ile Glu Glu
    290                 295                 300

Trp Met Ala Gly Val Ala Trp Leu His Cys Leu Leu Pro Lys Met Asp
305                 310                 315                 320

Ser Val Val Tyr Asp Phe Leu Lys Cys Met Val Tyr Asn Ile Pro Lys
                325                 330                 335

Lys Arg Tyr Trp Leu Phe Lys Gly Pro Ile Asp Ser Gly Lys Thr Thr
            340                 345                 350

Leu Ala Ala Val Leu Leu Glu Leu Cys Gly Gly Lys Ala Leu Asn Val
        355                 360                 365

Asn Leu Pro Leu Asp Arg Leu Asn Phe Glu Leu Gly Val Ala Ile Asp
    370                 375                 380

Gln Phe Leu Val Val Phe Glu Asp Val Lys Gly Thr Gly Gly Glu Ser
385                 390                 395                 400

Arg Asp Leu Pro Ser Gly Gln Gly Ile Asn Asn Leu Asp Asn Leu Arg
                405                 410                 415

Asp Tyr Leu Asp Gly Ser Val Lys Val Asn Leu Glu Lys Lys His Leu
            420                 425                 430

Asn Lys Arg Thr Gln Ile Phe Pro Pro Gly Ile Val Thr Met Asn Glu
        435                 440                 445

Tyr Ser Val Pro Lys Thr Leu Gln Ala Arg Phe Val Lys Gln Ile Asp
    450                 455                 460

Phe Arg Pro Lys Asp Tyr Leu Lys His Cys Leu Glu Arg Ser Glu Phe
465                 470                 475                 480

Leu Leu Glu Lys Arg Ile Ile Gln Ser Gly Ile Ala Leu Leu Leu Met
                485                 490                 495

Leu Ile Trp Tyr Arg Pro Val Ala Glu Phe Ala Gln Ser Ile Gln Ser
            500                 505                 510

Arg Ile Val Glu Trp Lys Glu Arg Leu Asp Lys Glu Phe Ser Leu Ser
```

        515   520   525

Val Tyr Gln Lys Met Lys Phe Asn Val Ala Met Gly Ile Gly Val Leu
    530   535   540

Asp Trp Leu Arg Asn Ser Asp Asp Asp Asp Glu Asp Ser Gln Glu Asn
545   550   555   560

Ala Asp Lys Asn Glu Asp Gly Gly Glu Lys Asn Met Glu Asp Ser Gly
                565   570   575

His Glu Thr Gly Ile Asp Ser Gln Ser Gln Gly Ser Phe Gln Ala Pro
            580   585   590

Gln Ser Ser Gln Ser Val His Asp His Asn Gln Pro Tyr His Ile Cys
        595   600   605

Arg Gly Phe Thr Cys Phe Lys Lys Pro Pro Thr Pro Pro Pro Glu Pro
    610   615   620

Glu Thr
625

<210> 9
<211> 57
<212> DNA
<213> simian virus 40

<400> 9
gatcccttca tcaggccatc tggccccttg ttaataatcg actgacccta ggtctaa   57

<210> 10
<211> 57
<212> DNA
<213> simian virus 40

<400> 10
gatcttagac ctagggtcag tcgattatta acaaggggcc agatggcctg atgaagg   57

<210> 11
<211> 57
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic -
      oligonucleotide

<400> 11
gatcccttca tcaggccatc tggccccttg ttaataatct aattacccta ggtctaa   57

<210> 12

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<211> 57
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic -
      oligonucleotide

<400> 12
gatcttagac ctagggtaat tagattatta acaaggggcc agatggcctg atgaagg     57


<210> 13
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic -
      oligonucleotide

<400> 13
ctcccaagct taagtgacca gctacaa                                      27


<210> 14
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic -
      oligonucleotide

<400> 14
gggcaaccta ggtactggac cttctatc                                     28


<210> 15
<211> 1022
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic -
      oligonucleotide

<400> 15
ggtaccgggc cccccctcga agacctaggg tcagtcgatt attaacaagg ggccagatgg 60

cctgatgaag ggatcttaga cctagggtca gtcgattatt aacaaggggc cagatggcct 120

gatgaaggga tcttagacct agggtcagtc gattattaac aaggggccag atggcctgat 180

gaagggatct tagacctagg gtcagtcgat tattaacaag gggccagatg gcctgatgaa 240
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gggatcttag acctagggtc agtcgattat taacaagggg ccagatggcc tgatgaaggg 300

atcttagacc tagggtcagt cgcttattaa caaggggcca catggcctga tgaagggatc 360

tcgatcgagg tcgatccagc tgagctaaga atccagctat caatagaaac tatgaaacag 420

ttccagggac aaagatacca ggtccccaac aactgcaact ttctgggaaa tgaggtggaa 480

aatgctcagc caaggaaaaa gagggcctta ccctctctgg gacaatgatt gtgctgtgaa 540

ctgcttcatc aggccatctg ccaaacttgt taataatcta attaccctag gtctaagtag 600

agttgttgac gtccaatgag cgctttctgc agacttagca ctaggcaagt gtttggaaat 660

tacagcttca gcccctctcg ccatctgcct acctacccct cctagagccc ttaatgggcc 720

aaacggcaaa gtccaggggc agagaggagg tgctttggac tataaagcta gtggagaccc 780

agtaactccc aagcttcgaa ttagcttaag tgaccagcta caatcataga ccatcagcaa 840

gcaggtatgt actctcctgg gtgagcccgg ttcccccagc caaaactcta gggactttag 900

gaaggatgtg ggttcctctc ttacatggac cttttcctag cctcaaccct gcctatcttc 960

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cc 1022

<210> 16
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic -
oligonucleotide

<400> 16
gagaaaggga attccatccc aaata 25

<210> 17
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic -
oligonucleotide

<400> 17
ttcaggatcc aaggatcagc agg 23

<210> 18

<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic -
oligonucleotide

<400> 18
ggggaactag taaactcttt gttgaag 27

<210> 19
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic -
oligonucleotide

<400> 19
ctcagtaact cgagttaatg aagtcct 27